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(54) Title: INDUCTION OF CYTOTOXIC T-LYMPHOCYTE RESPONSES

(57) Abstract

Methods and compositions useful for inducing a cytotoxic T-lymphocyte response (CTL) in a human or domesticated or agriculturally important animal. The method includes the steps of providing the antigen to which the CTL response is desired and providing an antigen formulation which comprises, consists, or consists essentially of two or more of a stabilizing detergent, a micelle-forming agent, and an oil. This antigen formulation is preferably lacking in an immunostimulating peptide component, or has sufficiently low levels of such a component that the desired CTL response is not diminished. This formulation is provided as a stable oil-in-water emulsion.

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INDUCTION OF CYTOTOXIC T-LYMPHOCYTE RESPONSES

Background of the Invention

Pending U.S. Serial No. 08/919,787 filed July 24, 1992; and U.S. Serial No. 07/735,069, filed July 25, 1991, both entitled "Induction of Cytotoxic T-Lymphocyte Responses," by Syamal Raychaudhuri and William H. Rastetter (now abandoned), are incorporated by reference in their entirety in the present application. This invention relates to methods and compositions useful for inducing cytotoxic T-cell mediated responses in humans, and domesticated or agricultural animals.

Cytotoxic T-lymphocytes (CTLs) are believed to be the major host defense mechanism in response to a variety of viral infections and neoplastic or cancerous growth. These cells eliminate infected or transformed cells by recognizing antigen fragments in association with various molecules (termed class I MHC molecules) on the infected or transformed cells. CTLs may be induced experimentally by cytoplasmic loading of certain soluble antigens within specific cells. Immunization with the soluble antigen alone is generally insufficient for specific cytotoxic T-lymphocyte induction.

One method by which CTL response may be induced involves the use of recombinant engineering techniques to incorporate critical components of an antigen in question into the genome of a benign infectious agent. The aim of such a strategy is to generate antigen-specific cytotoxic T-lymphocyte responses to the desired epitope by subjecting the host to a mild, self-limiting infection. Chimeric vectors have been described using vaccinia, polio, adeno- and retro-viruses, as well as bacteria such as Listeria and

BCG. For example, Takahashi et al. 85 Proc. Natl. Acad. Sci., USA 3105, 1988 describe use of recombinant vaccinia virus expressing the HIV gp160 envelope gene as a potential tool for induction of cytotoxic T-lymphocytes.

A second method by which a cell mediated response may be induced involves the use of adjuvants. While the art appears replete with discussion of the use of adjuvants, it is unclear in such art whether cell mediated immunity was induced and whether such cell mediated immunity included a cytotoxic T-lymphocyte response. The following, however, are representative of various publications in this area.

Stover et al., 351 Nature 456, 1991 (not admitted to be prior art to the present application) describes a CTL response to β-galactosidase using recombinant BCG containing a β-galactosidase gene. No such response was detected using incomplete Freund's adjuvant and β-galactosidase.

Mitchell et al., 8 J. Clinical Oncology 856, 1990 (which is not admitted to be prior art to the present invention) describe treatment of metatastic melanoma patients with an adjuvant termed "DETOX" and allogeneic melanoma lysates administered five times over a period of six weeks. In a small portion of the patients an increase in cytolytic T-cells was observed. The authors describe a need to enhance the level of cytotoxic T-lymphocyte production, and suggest a combined therapy of adjuvant with Interleukin-2, as well as a pretreatment with cyclophosphamide to diminish the level of tumor specific T-suppressor cells that might exist. DETOX includes detoxified endotoxin (monophosphoryl lipid A) from *Salmonella minnesota*, cell wall skeletons of *Mycobacterium phlei*, squalene oil and emulsifier.

Allison and Gregoriadis, 11 Immunology Today 427, 1990 (which is not admitted to be prior art to the present invention) note that the only adjuvant "authorized for use" in human vaccines is aluminum salts (alum) which does not consistently elicit cell mediated immunity. Allison and Gregoriadis state "[t]here is, therefore, a need to develop adjuvants with the efficacy of Freund's complete adjuvant but without its various side effects such as granulomas." They go on to state that three possible strategies exist, for example, the use of liposomes; the use of adjuvants, termed immunostimulating complexes (ISCOMs, which include saponin or Quil A (a triterpenoid with two carbohydrate chains), cholesterol, and phosphatidyl choline) which are authorized for use in an influenza vaccine for horses (Morein et al., Immunological Adjuvants and Vaccines, Plenum Press, 153); and the use of an emulsion (SAF) of squalene or Squalane (with or without a pluronic agent) and muramyl dipeptide (MDP). SAF is said to elicit a cell mediated immunity in mice, although it "has long been thought that subunit antigens cannot elicit cytotoxic T-cell (CTL) responses."

Takahashi et al., 344 Nature 873, 1990, describe class II restricted helper and cytotoxic T-lymphocyte induction by use of ISCOMs with a single subcutaneous immunization in mice. They state that Freund's adjuvant, incomplete Freund's adjuvant, and phosphate buffered saline did not induce cytotoxic T-lymphocyte activity against the targets in which they were interested. They state that, in contrast to results with other forms of exogenous soluble protein antigen, they have shown that it is possible to prime antigen specific MHC class I restricted $CD8^+$ $CD4^-$ CTL by immunization with exogenous intact protein using ISCOMs. They also state that the experiments described suggest that it may be possible to elicit human CTL by using ISCOMs

containing HIV proteins, and that ISCOM-based vaccines may achieve the long sought goal of induction of both CTL and antibodies by a purified protein.

Byars and Allison, 5 Vaccines 223, 1987 describe use of SAF-1 which includes TWEEN 80, PLURONIC L121, and squalene or Squalane, with or without muramyl dipeptide, and suggest that their data indicate that the formulation with muramyl dipeptide will be useful for human and veterinary vaccines. Booster shots of the adjuvant were provided without the muramyl dipeptide. The muramyl dipeptide is said to increase antibody production significantly over use of the adjuvant without muramyl dipeptide. Cell mediated immunity was measured as delayed type hypersensitivity by skin tests to determine T-helper cell induction. Such hypersensitivity was stronger and more sustained when muramyl dipeptide was provided in the adjuvant. Similar adjuvants are described by Allison et al., U.S. Patent 4,770,874 (where it is stated that the combination of muramyl dipeptide and pluronic polyol is essential to elicit a powerful cell mediated and humoral response against egg albumin); Allison et al., U.S. Patent 4,772,466; Murphy-Corb et al., 246 Science 1293, 1989 (where it is stated that the use of combined adjuvants with muramyl dipeptide might enhance induction of both humoral and cellular arms of the immune response); Allison and Byars, 87 Vaccines 56, 1987 (where it is stated that cell mediated immunity is elicited by SAF (with muramyl dipeptide) as shown by delayed type hypersensitivity, by proliferative responses of T-cells to antigen, by production of Interleukin-2, and by specific genetically restricted lysis of target cells bearing the immunizing antigen); Allison and Byars, Immunopharmacology of Infectious Diseases: Vaccine Adjuvants and Modulators of Non-Specific Resistance 191-201, 1987; Morgan et al., 29 J.

Medical Virology 74, 1989; Kenney et al., 121 J. Immunological Methods 157, 1989; Allison and Byars, 95 J. Immunological Methods 157, 1986 (where aluminum salts and mineral oil emulsions were shown to increase antibody formation, but not cell mediated immunity; and muramyl dipeptide formulations were shown to elicit cell mediated immunity); Byars et al., 8 Vaccine 49, 1990 (not admitted to be prior art to the present application, where it is stated that their adjuvant formulation markedly increases humoral responses, and to a lesser degree enhances cell mediated reactions to influenzae haemagglutinin antigen); Allison and Byars, 28 Molecular Immunology 279, 1991 (not admitted to be prior art to the present application; which states that the function of the muramyl dipeptide is to induce expression of cytokines and increase expression of major histocompatibility (MHC) genes; and that better antibody and cellular responses were obtained than with other adjuvants, and that it is hoped to ascertain whether similar strategies are efficacious in humans); Allison and Byars, Technology Advances in Vaccine Development 401, 1988 (which describes cell mediated immunity using SAF); Epstein et al., 4 Advance Drug Delivery Reviews 223, 1990 (which provides an overview of various adjuvants used in preparation of vaccines); Allison and Byars, 95 J. Immunological Methods 157, 1986 (which states that the addition of the muramyl dipeptide to the adjuvant markedly augments cell mediated responses to a variety of antigens, including monoclonal immunoglobulins and virus antigens); and Morgan et al., 29 J. Medical Virology 74, 1989 (which describes use of SAF-1 for preparation of a vaccine for Epstein-Barr virus).

Kwak et al., Idiotype Networks in Biology and Medicine, Elsevier Science Publishers, p. 163, 1990 (not admitted to be prior art to the present application)

describe use of SAF without muramyl dipeptide as an adjuvant for a B-cell lymphoma idiotype in a human. Specifically, an emulsion of Pluronic L121, Squalane, and 0.4% TWEEN-80 in phosphate buffered saline was administered with the idiotype. They state that "[a]ddition of an adjuvant should further augment ... humoral responses, and may facilitate induction of cellular responses as well.

Other immunological preparations include liposomes (Allison et al., U.S. Patents 4,053,585, and 4,117,113); cyclic peptides (Dreesman et al., U.S. Patent 4,778,784); Freunds Complete Adjuvant (Asherson et al., 22 Immunology 465, 1972; Berman et al., 2 International J. Cancer 539, 1967; Allison, 18 Immunopotentiation 73, 1973; and Allison, Non-Specific Factors Influencing Host Resistance 247, 1973); ISCOMs (Letvin et al., 87 Vaccines 209, 1987); adjuvants containing non-ionic block polymer agents formed with mineral oil, a surface active agent and TWEEN 80 (Hunter and Bennett, 133 J. Immunology 3167, 1984; and Hunter et al., 127 J. Immunology 1244, 1981); adjuvants composed of mineral oil and emulsifying agent with or without killed mycobacteria (Sanchez-Pescador et al., 141 J. Immunology 1720, 1988); and other adjuvants such as a lipophilic derivative of muramyl tripeptide, and a muramyl dipeptide covalently conjugated to recombinant protein (id.).

Summary of the Invention

Applicant has discovered a safe and advantageous method and compositions by which CTL responses may be induced in humans and domesticated or agriculturally important animals. The method involves the use of an antigen formulation which has little or no toxicity to animals, and lacks an immunostimulating peptide, (e.g., muramyl dipeptide) the presence of which would decrease the desired cellular response. In addition, the methodology is

simple to use and does not require extensive in vivo work to alter existing cells by recombinant DNA techniques to make them more antigenic. This discovery is surprising since it was unexpected that such a CTL response could be induced by use of such an antigen formulation lacking immunostimulating peptides or their equivalent. Applicant's findings allow the use of such antigen formulations in a broad spectrum of disease states, or as a prophylactic agent. For example, such antigen formulation administration can be used for the treatment of viral diseases in which a CTL response is important, for example, in the treatment of HIV infection or influenza; it can also be extended to use in treatment of bacterial infections, cancer, parasitic infections, and the like. As a prophylactic agent, the antigen formulation combined with a suitable antigen is useful in prevention of infection by viruses responsible for the aforementioned viral diseases, particularly the prophylaxis of HIV infection, and also for prophylaxis of patients at risk of cancer, for example, after resection of a primary tumor.

Thus, in a first aspect the invention features a method for inducing a CTL response in a human or domesticated (e.g., a cat or dog) or agriculturally important animal (e.g., a horse, cow or pig) to an antigen other than B-cell lymphoma antigen or egg albumin. The method includes the steps of providing the antigen to which the CTL response is desired, and providing a non-toxic antigen formulation which comprises, consists, or consists essentially of, a stabilizing detergent, a micelle-forming agent, and a biodegradable and biocompatible oil. This antigen formulation preferably lacks any immunostimulating peptide component, or has sufficiently low levels of such a component that the desired cellular response is not diminished. This formulation is preferably provided as a

stable oil-in-water emulsion. That is, each of the various components are chosen such that the emulsion will remain in an emulsion state for a period of at least one month, and preferably for more than one year, without phase separation. In the method the antigen and antigen formulation are mixed together to form a mixture (preferably by microfluidization), and that mixture administered to the animal in an amount sufficient to induce CTL response in the animal. Such administration is required only once.

By "stabilizing detergent" is meant a detergent that allows the components of the emulsion to remain as a stable emulsion. Such detergents include polysorbate, 80 (TWEEN) (Sorbitan-mono-9-octadecenoate-poly(oxy-1,2-ethanediyl; manufactured by ICI Americas, Wilmington, DE), TWEEN 40, TWEEN 20, TWEEN 60, Zwittergent 3-12, TEEPOL HB7, and SPAN 85. These detergents are usually provided in an amount of approximately 0.05 to 0.5%, preferably at about 0.2%.

By "micelle-forming agent" is meant an agent which is able to stabilize the emulsion formed with the other components such that a micelle-like structure is formed. Such agents preferably cause some irritation at the site of injection in order to recruit macrophages to enhance the cellular response. Examples of such agents include polymer surfactants described by BASF Wyandotte publications, e.g., Schmolka, 54 J. Am. Oil. Chem. Soc. 110, 1977, and Hunter et al., 129 J. Immunol 1244, 1981, both hereby incorporated by reference, PLURONIC L62LF, L101, and L64, PEG1000, and TETRONIC 1501, 150R1, 701, 901, 1301, and 130R1. The chemical structures of such agents are well known in the art. Preferably, the agent is chosen to have a hydrophile-lipophile balance (HLB) of between 0 and 2, as defined by Hunter and Bennett, 133 Journal of

Immunology 3167, 1984. The agent is preferably provided in an amount between 0.5 and 10%, most preferably in an amount between 1.25 and 5%.

The oil is chosen to promote the retention of the antigen in oil-in-water emulsion, i.e., to provide a vehicle for the desired antigen, and preferably has a melting temperature of less than 65°C such that emulsion is formed either at room temperature (about 20°C to 25°C), or once the temperature of the emulsion is brought down to room temperature. Examples of such oils include squalene, Squalane, EICOSANE, tetratetracontane, glycerol, and peanut oil or other vegetable oils. The oil is preferably provided in an amount between 1 and 10%, most preferably between 2.5 and 5%. It is important that the oil is biodegradable and biocompatible so that the body can break down the oil over time, and so that no adverse affects, such as granulomas, are evident upon use of the oil.

It is important in the above formulation that a peptide component, especially a muramyl dipeptide (MDP) be lacking. Such a peptide will interfere with induction of a CTL response if it provided in an amount greater than about 20 micrograms per normal human formulation administration. It is preferred that such peptides be completely absent from the antigen formulation, despite their apparent stimulation of the humoral compartment of the immune system. That is, applicant has found that, although such peptides may enhance the humoral response, they are disadvantageous when a cytotoxic T-lymphocyte response is desired.

In other related aspects, the antigen formulation is formed from only two of the above three components and used with any desired antigen (which term includes proteins, polypeptides, and fragments thereof which are immunogenic) except egg albumin (or other albumins, e.g.,

HSA, BSA and ovalbumin), to induce a CTL response in the above animals or humans.

Applicant believes that the above formulations are significantly advantageous over prior formulations (including ISCOMs, DETOX, and SAF) for use in humans. Unlike such formulations, the present formulation both includes a micelle-forming agent, and has no peptides, cell wall skeletons, or bacterial cell components. The present formulation also induces a CTL response which either does not occur with the prior formulations, or is significantly enhanced compared to those formulations.

By "non-toxic" is meant that little or no side effect of the antigen formulation is observed in the treated animal or human. Those of ordinary skill in the medical or veterinary arts will recognize that this term has a broad meaning. For example, in a substantially healthy animal or human only slight toxicity may be tolerated, whereas in a human suffering from an imminently disease substantially more toxicity may be tolerated.

In preferred embodiments, the antigen formulation consists essentially of two or three of the detergent, agent, and oil; the method consists essentially of a single administration of the mixture (antigen plus antigen formulation) to the human or the animal; the human or animal is infected with a virus and suffers one or more symptoms (as generally defined by medical doctors in the relevant field) of infection from the virus; and the antigen formulation is non-toxic to the human or animal.

In other preferred embodiments, the antigen is chosen from antigenic portions of the HIV antigens: gp160, gag, pol, Nef, Tat, and Rev; the malaria antigens: CS protein and Sporozoite surface protein 2; the Hepatitis B surface antigens: Pre-S1, Pre-S2, HBc Ag, and HBe Ag; the influenza antigens: HA, NP and NA; Hepatitis A surface

antigens; the Herpes virus antigens: EBV gp340, EBV gp85, HSV gB, HSV gD, HSV gH, HSV early protein product, human papillomavirus antigens (e.g., HPV antigens, such as L1, E4, E6, E7 antigens, in particular the E6 and E7 antigens from HPV16 and 18, the two most common HPV types associated with cervical carcinoma, E4 and L1 derived from HPV6 and HPV11, the two most common HPV types associated with condyloma acuminata; the prostate specific antigen (PSA), cytomegalovirus gB, cytomegalovirus gH, and IE protein gP72; the respiratory syncytial virus antigens: F protein, G protein, and N protein; and the tumor antigens carcinoma CEA, carcinoma associated mucin, carcinoma P21, carcinoma P53, melanoma MPG, melanoma p97, and carcinoma Neu oncogene product, carcinoma p53 gene product, the melanoma antigen called MAGE, and mutated p21 ras protein presented in a variety of malignant tumors.

In related aspect, the invention features a composition comprising, consisting, or consisting essentially of an antigen mixed with an antigen formulation described above, and the antigen is chosen from those antigenic portions listed above.

In other related aspects, the invention features methods of treating a patient infected with HIV virus, suffering from malaria, suffering from influenza, suffering from hepatitis, suffering from a cancer, infected with herpes virus, suffering from cervical cancer, suffering from condyloma acuminata (genital warts), or infected with respiratory syncytial virus, by administering a composition including an appropriate antigen (e.g., selected from those listed above) mixed with one of the above antigen formulations. These antigens and treatments are only exemplary of antigens which may be used in the subject antigen formulations.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

FIGS. 1A - 1C and 4A - 4C are graphical presentations of data comparing CTL induction by various ovalbumin formulations; E:T represents effector to target ratio in all Figures.

FIGS. 2A and 2B are graphical presentations of data comparing CTL induction by various β-galactosidase formulations;

FIG. 3 is a graphical presentation of data comparing CTL induction by ovalbumin in a liposome and in an antigen formulation;

FIGS. 5 and 6 are graphical presentations of data showing the effect of CD4 and CD8 cell depletion on CTL induction;

FIG. 7 is a graphical representation of data showing CTL induction by gp120;

FIG. 8 is a graphical representation of data showing CTL induction by a mixture of pluronic and TWEEN and an antigen;

FIG. 9 is a graphical representation of data showing CTL induction with a mixture of squalane and pluronic and an antigen;

FIG. 10 is a graphical representation of data showing CTL induction by a mixture of squalane and pluronic and an antigen;

FIG. 11 is a graphical representation of the effect of OVA with various antigen formulations on CTL response;

FIG. 12 is a graphical representation of the induction of anti-gp120IIIb antibodies in monkeys with various antigen formulations;

FIG. 13 depicts antitumor activity of HOPE2 cells ten days after a single immunization of soluble E7 protein in adjuvant; and

FIG. 14 depicts antitumor activity of HOPE2 cells at days 10, 19 after two immunizations with soluble E7 protein in adjuvant.

<u>Antigen Formulation</u>

Antigen formulations useful in this invention are generally described above. Those of ordinary skill in this art will recognize that equivalent Formulations are readily prepared and can be expected to have equivalent properties in induction of a CTL response. Such Formulations are readily tested for their properties using techniques equivalent to those described in the examples below.

There follow examples of the invention with the use of an antigen formulation (AF) composed of about 2.5% squalane, 5% pluronic acid, and TWEEN 80 in a phosphate buffered saline. Specifically, an emulsion of the AF included : 15 mg squalane, 37.5 mg poloxamer 401 (PLURONIC L121), 6 mg polysorbate 80 (TWEEN 80), 0.184 mg potassium chloride, 0.552 mg potassium phosphate monobasic, 7.36 mg. sodium chloride, 3.3 mg sodium phosphate dibasic (anhydrous), per 1 ml water, pH 7.4. This emulsion was microfluidized using standard technique (Microfluidics Model M110F) with a back-pressure module at 11-14,000 psi with gradual return to atmosphere pressure, cooling and packing in wet ice.

In other examples, antigen was mixed with the microfluidized squalane (S), pluronic (P) and TWEEN 80 (T) mixture to achieve a final concentration of 0.2% TWEEN 80,

1.25% pluronic and 5% squalane respectively. To determine the sub-components necessary for an antigen specific immune response induction, Squalane-TWEEN 80, pluronic-TWEEN 80 or Squalane-pluronic were prepared at the same concentration as for the three components mixture. Pluronic, Squalane or TWEEN 80 was also prepared individually to determine the effect of individual component on the CTL induction. Substitutions of TWEEN 20, TWEEN 40 or Zwittergent for TWEEN 80 were also made to determine the effect of various TWEEN derivative on the CTL induction in the *ova* system. Substitutions of Squalane in the three component formulation were made with Eicosone or Triacontone and substitution for the co-polymer pluronic in the same three components formulation were made by PEG 1000, Pleuronic L62LF, and the Tetronics 1501 and 150R1. As two component formulations, various analogs in various combinations were mixed and tested for *ova* specific CTL induction. They are a mixture of cholesterol - TWEEN 80, Squalane - TWEEN 20, Pristane - TWEEN 80 or olive oil - TWEEN 80. For a stabilization study, the microfluidized mixture of Squalane-TWEEN 80 was mixed with dextrose to a final concentration of 5%. In all cases the combinations of excipients were mixed in a microfluidizer to made a stable emulsion. In some experiments, two components formulations were mixed with various concentration of MDP for CTL and humoral response inductions. Table 1 describes a comprehensive list of various formulations used in this study.

Table 1

Effect of various substitution in three or two component systems

Substitution in three component formulations	percent kill at E:T 100:1
STP	84
Tween 40(T)	66
Tween 20(T)	48
T1501(P)	0
T150R1(P)	0
Pluronic L62LF(P)	47
Eicosane(S)	*
PEG1000(P)	*
Triacontane(S)	*
Zwittergent(T)	*
Substitution in two component formulations	
ST	76
PT	45
SP	26
Cholesterol(S) + Tween 80	0
Squalane + Tween 29(T)	65
Pristane(S) + Tween 80	42
Olive Oil(S) + Tween 80	69
1 component formulation	
Pluronic L121	0
Squalane	0
Tween 80	0
Squalane + Tween 80 + 5% dextrose	86

* CTL assay is being repeated

Syntex adjuvant formulation (microfluidized; SAFm) was used as an adjuvant control and consists of two parts. Part I consists of phosphate buffered saline containing a final concentration of 5% Squalane, 1.25% pluronic and 0.2% TWEEN 80 (vehicle or I-SAF). Part II consists of N-Acetylmuramyl-L-Threonyl-D-Isoglutamine (Thr-MDP), a derivative of mycobacterium cell wall component. For immunization purposes, antigen is mixed with

microfluidized vehicle (part I) to obtain a homogeneous emulsion. MDP is added to made SAFm, and vortexed briefly. The MDP concentration in the mixture was varied to determine if there was an optimum concentration for CTL induction. As an adjuvant control, mice were also immunized with soluble antigens mixed with alum according to the manufacturer's manual (Pierce Chemical, Rockford, IL) or with Complete Freund's Adjuvant (CFA).

This antigen formulation is used for induction of cytotoxic T-lymphocyte responses in mice. Those of ordinary skill in the art will recognize that such a mouse model is indicative that equivalent experiments or treatments will similarly induce cytotoxic T-lymphocyte responses in humans, domesticated, or agricultural animals. The amount of antigen formulation and antigen useful to produce the desired cellular response may be determined empirically by standard procedures, well known to those of ordinary skill in the art, without undue experimentation. Thus, if desired to minimize the side effects of treatment with such a mixture those of ordinary skill in the art may determine a minimum level of such a mixture for administration to a human, domesticated, or agricultural animal in order to elicit a CTL response, and thereby induce immunity to a desired antigen. In normal use, such a mixture will be injected by any one of a number of standard procedures, but particularly preferred is an intramuscular injection at a location which will allow the emulsion to remain in a stable form for a period of several days or several weeks.

Methods

The following materials and methods were used in the examples provided below unless otherwise noted:

Mice

Female C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from Harlen Sprague (San Diego, California).

Antigens

Ovalbumin (*ova*, Grade VII; Sigma Chemical Co., St. Louis, MO) was used in the native form. β-galactosidase, (β-gal, Grade VIII; BRL) was used in the native form and after boiling in 1 M NaOH for 2 min to give an alkali digest. Recombinant gp120 was purchased from American Biotechnology.

Tumor Cells and Transfectants

The tumor cells used were the Ia^- lines EL4 (C57BL/6, H-2^b thymoma) and P815 (DBA/2, H-2^d mastocytoma). Derivation of the *ova*-producing EL4 transfectant, EG7-*ova*, is described previously by Moore et al., 54 Cell 777, 1988. The β-gal-producing transfectant, P13.1, was derived by electroporation of 10^7 P815 cells in 1 ml of phosphate buffered saline (PBS) with 10 mg of PstI linearized pCH110 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) and 1 mg of PvuI linearized pSV2 neo (Southern et al., 1 J. Mol. Appl. Genet. 327, 1982) followed by selection in 400 μg/ml of the antibiotic G418. The C3-4 transfectant was derived from the BALB/c hybridoma Igm 662 by transfecting with a plasmid encoding the β-gal gene fused to the third and fourth exon of IgM heavy chain (Rammensee et al., 30 Immunogenetics 296, 1989). The gp160IIIb expressing 3T3 fibroblast, 15-12, was provided by Dr. Germain of NIH (Bethesda, MD). The K^b transfected L cell line was provided by Dr. Carbone, Monash University, Australia. The D^d and L^d transfected L cell lines were provided by Dr. Ted Hensen, Washington University, St. Louis.

Immunization

Mice were immunized intravenously with a 200 μl suspension of 25 x 10^6 splenocytes, after a cytoplasmic loading as described by Moore et. al. supra, and Carbone et al., J. Exp. Med. 169:603, 1989). For *ova*-antigen formulation or β-gal-antigen formulation immunization, 30 μg of each protein antigen was injected per mouse in the footpad and the tailbase subcutaneously. Each injection consists of 67 μl of microfluidized antigen formulation (made following standard procedures) and 30 μg of protein antigen in a final volume of 200 μl. The final volume was made up with HBSS, see, Whittaker manual (Welkersville, MD). MDP was provided in concentrations between 0 and 300 μg. Where stated, mice were immunized with soluble antigens in CFA, or in alum in a total volume of 200 μl.

In vitro stimulation of effector populations

Spleen cells (30 x 10^6) from normal or immunized mice which had been primed at least 14 days earlier were incubated with 1.5 x 10^6 EG7-*ova* (irradiated with 20,000 rad) for *ova* responses or 1.5 x 10^6 C3-4 cells (irradiated with 20,000 rad) for β-gal response in 24 well plates at 37°C in 7% CO_2/air. All the tissue cultures were performed in a complete medium consisting of IMDM medium, see, Whittaker Manual (Welkersville, MD) supplemented with 10% fetal calf serum (FCS), 2mM glutamine, gentamycin and 2 x 10^{-5} M 2-mercaptoethanol. For the *in vitro* depletion experiments, *in vivo* primed or *in vitro* stimulated spleen cells were treated with monoclonal antibodies (mAbs) RL.172 (anti-CD4) or mAbs 3.168 (anti-CD8) for removal of $CD4^+$ or $CD8^+$ T cells (Sarmiento et al., 125 J. Immunol. 2665, 1980, and Ceredig et al., 314 Nature 98, 1985). The mAb RL.172 and mAb 3.168 were obtained from Dr. Jonathan Sprent at Scripps Clinic and Research Foundation, La Jolla, CA.

Spleen cells (30×10^6) from normal or immunized mice which had been primed at least 21 days earlier were incubated with 1.5×10^6 15-12 cells (treated with 200ug of mitomycin C for 45 minutes per 10^8 cells), or with 500 μg of 18IIIb peptide containing the dominant CTL epitope in Balb/c mice in complete IMDM media (Irvine Scientific, Santa Ana, CA) containing 10% pre-screened FCS (ICN Flow; ICN Biochemicals, Inc., Costa Mesa, CA), 2mM glutamine, gentamycin and 2×10^{-5} M 2-mercaptoethanol. For in vitro stimulation with peptides, spleen cells were cultured in complete IMDM containing 5% ConA supernatant.

For depletion experiments, in vivo primed or in vitro stimulated spleen cells were treated with mAbs RL.172 (anti-CD4) or mAbs 3.168 (anti-CD8) in presence of low tox. rabbit complement (Cederlane Laboratories, Ltd., Hornby Ontario, Canada) for removal of $CD4^+$ or $CD8^+$ T cells (22, 23). The mAb RL.172 and mAb 3.168 were a gift from Dr. Jonathan Sprent at Scripps Clinic and Research Foundation, La Jolla, CA.

Cytoxicity Assay

Target cells (1×10^6) were labeled with 100 μCi [^{51}Cr] sodium chromate for 60 min. For peptide pulsed targets, 50 μl of a 1 mg/ml peptide solution in HBSS was added during the targets labeling with ^{51}Cr. After washing, 10^4 labeled targets and serial dilutions of effector cells were incubated in 200 μl of RP10 for 4 h at 37°C. 100 μl of supernatant was collected and the specific lysis was determined as: Percent specific lysis = 100 x {(release by CTL - spontaneous release)/(maximal release - spontaneous release)}. Spontaneous release in the absence of cytotoxic T-lymphocyte (CTL) was <25% of maximal release by detergent in all experiments.

Determination of Antibody Responses in Mice and Monkeys

Each well of 96-well, U bottomed plates (Costar, Cambridge, MA) were coated with 150 ng of *ova* or gp120 in 50 ul of HBSS and incubated overnight at 4°C. For the determination of anti-gp120 and anti-*ova* antibody responses in mice, plates were blocked with 1% BSA for 1 hr. Serially diluted sera were added in 25 μl volume per well and incubated for 2 hrs. Plates were washed and 50 μl of 1:1000 dilution of goat anti-mouse IgG conjugated to HRPO (SBT, Alabama) in 1% BSA were added per well. After 1 hr of incubation, plates were washed and 100 μl of substrate was added per well. The OD_{405} was taken after 10 to 15 minutes. For the determination of monkey anti-gp120 antibody response, all the steps were the same except both the blocking of plates and the dilution of sera were done in 5% normal goat serum in Hank's balanced salt solution.

Peptide Synthesis

Synthetic peptides corresponding to amino acid sequences 253-276 (Sequence Listing No. 1: EQLESIINFEKLTEWTSSNVMEER; where the standard one letter code is used to represent each amino acid) of ovalbumin (*ova* 253-276), amino acid sequences 84-102 of myelin basic protein (MBP 84-102) (Sequence Listing No. 2: DENPVVHFFKNIVTPRTPP), and synthetic peptides corresponding to amino acid sequences 308-322 (18IIIb sequence) of gp120IIIb, were assembled by solid phase peptide synthesis using an Applied Biosystems 430A synthesizer. Amino acids were coupled via pre-formed symmetric anhydrides with the exception of asparagine, glutamine and arginine which were coupled as hydroxybenzotriazole esters. Coupling efficiency was monitored by ninhydrin reaction following the method of Kaiser et al. 34 Anal. Biochem. 595, 1970. The peptides were released from the support with HF

following the "low-high" procedure described by Tam, et al. 21 J. Am. Chem. Soc. 6442, 1983, and the peptides extracted from the resin with 10% acetic acid. After lyophilization, peptides were desalted on a Sephadex G-25 column, and samples of the peptides then HPLC purified by reverse phase chromatography on a Vydac preparative C-18 column. Purified peptides (98%) were solubilized in HBSS at a final concentration of 10 mg/ml and diluted to the desired concentration in the complete media.

CNBr Digest

Samples of protein (e.g., β-galactosidase) were treated with 100 fold molar excess of cyanogen bromide in a solution of 100 mM trifluoroacetic acid. The reaction was allowed to proceed for 18 hours at room temperature (about 20°C) with rotation. Following the prescribed reaction time, the peptide fragments were separated from the reactant using a SEP-PAK C-18 apparatus (Waters), eluted with 95% acetonitrile, and lyophilized.

Alkaline digest

Protein samples (e.g., β-galactosidase) were treated with 1 N NaOH and boiled for 2 minutes, and the resulting peptide fragments were separated from the reactants using a C-18 SEP-PAK apparatus (Waters), and eluted with 95% acetonitrile and lyophilized.

Example 1: Class I restricted CTL priming

Moore et al., 113 UCLA Symp. Mol. Cell. Biol. 1989 and Carbone and Bevan, 171 J. Exp. Medicine 377, 1990, demonstrate that mice immunized with spleen cells loaded cytoplasmically with soluble *ova*, were primed for *ova* specific, class I restricted CTL response. The *ova*-expressing EL4 transfectant EG7-*ova* was employed for *in vitro* stimulation of *in vivo* primed splenic lymphocytes and also used as target for *ova* specific CTL mediated killing. This study also demonstrated that $CD8^+$ effectors induced by

EG7-*ova* transfectant or by spleen cells cytoplasmically loaded with *ova*, recognize a determinant mapped by the peptide *ova* 258-276 in the context of H-2K^b, lyse EG7-*ova*, and also kill EL4 cells coated with *ova* 258-276. Thus, in order to assess whether an endogenous class I restricted CD8$^+$ T cell pathway can be induced by a soluble antigen, the above system was used to determine whether certain antigen formulations can be used to drive soluble antigen into a class I restricted pathway.

a) *ova*

C57BL/6 mice were immunized once with various amounts of *ova* (30 μg - 1 mg per mouse) with or without an antigen formulation. Mice were injected subcutaneously and in the tailbase. Spleen cells were taken from the immunized mice at least two weeks after the immunizations and *in vitro* stimulated with the EG7-*ova* transfectants. An *ova* concentration as low as 30 μg was as effective as a 1 mg dose. Therefore, the CTL studies were routinely performed with spleen cells from 30 μg *ova*-primed mice. After five days of *in vitro* culture with EG7-*ova*, priming was assessed by the presence of *ova* specific effectors capable of lysing EG7-*ova*.

Mice injected with soluble *ova* in HBSS as high as 1 mg, showed no evidence of CTL priming (FIG. 1A). However mice immunized with 30 μg *ova* in the antigen formulation described above (shown as AF in the figures) showed a significant transfectant specific CTL response (FIG. 1C). Furthermore, the extent of EG7-*ova* killing by the *ova*-AF immunized spleen cells was comparable to that of *ova*-loaded spleen cells immunized mice (FIG. 1B).

That the specificity of CTL priming in vivo was antigen specific was shown by the lack of spleen cells from β-galactosidase immunized mice to manifest secondary CTL

response *in vitro* when stimulated with EG7-*ova*. No *ova* specific CTL induction was observed.

b) β-galactosidase

Similar results were obtained using another soluble protein antigen, β-gal. For assaying β-gal-specific CTL response, the target used was BALB/c derived β-gal-expressing C3-4 transfectant. Immunization of BALB/c mice with soluble β-gal gave background CTL response. Therefore, for the determination of specific CTL response, harvesting was postponed for at least eight weeks before spleen lymphocytes were harvested, and cultured for five days in the presence of irradiated C3-4 transfectants.

FIG. 2B demonstrates that 30 μg of β-galactosidase in AF induced strong specific CTL response against transfectant. At an effector-to-target (E:T) ratio of 3:1, β-gal-AF immunized mice showed about 80% of specific C3-4 killing. However, only 20% killing of the same target was achieved with effectors isolated from β-gal in HBSS immunized mice at the same E:T ratio (FIG. 2A). Since neither EL4 nor P815 expresses class II MHC gene products and the lysis shows syngeneic restriction, these *ova* and β-gal specific effectors are class I MHC restricted.

To demonstrate the usefulness of the antigen formulation, mice were immunized with soluble *ova* encapsuled in two types of liposomes, one of which was a pH sensitive liposome. One week later, spleen cells were stimulated in vitro, as described above, and tested against ^{51}Cr-labeled EG7-*ova* or EL4. FIG. 3 shows a representative result demonstrating that *ova* in liposome could not prime mice for substantial CTL induction. Similar results were observed when *ova* was immunized in alum.

Example 2: Recognition of epitope by CTL

Carbone and Bevan, supra, demonstrated that CTL induced in C57BL/6 mice by EG7-*ova* transfectant, and by cytoplasmically *ova*-loaded splenocytes recognize EL4 cells coated with the peptide *ova* 258-276. To determine whether soluble ovalbumin in AF induces similar CTL responses, spleen cells were prepared from immunized mice and stimulated *in vitro* with EG7-*ova*. The effectors were tested against EL4 cells coated with the peptide *ova* 253-276, or with a control peptide derived from myelin basic protein (MBP 84-102). The results demonstrate that *ova*-AF primed CTL with a similar specificity to those primed by transfectants, or by cytoplasmically loaded *ova* (FIGS. 1A, 1B and 1C). *ova*-AF primed effector cells effectively lysed EG7-*ova*, and an untransfected EL4 cells coated with 50 $\mu g/10^8$ cells of *ova* peptide, but did not lyse EL4 cells coated with 50 $\mu g/10^8$ cells of MBP peptide.

In the β-galactosidase system, Carbone and Bevan, supra, indicated that β-gal expressing transfectant and splenocytes cytoplasmically loaded with soluble β-galactosidase, induced CTL which lysed β-gal expressing transfectant and nontransfectant P815 cells coated with alkali digested β-galactosidase. Soluble β-galactosidase induces CTL having similar specificity when immunized in AF (FIG. 2).

Example 3: CTL effectors are $CD8^+$ T cells

That soluble protein antigens in AF induce $CD8^+$ effector T cells was shown as follows. Splenocytes from immunized mice were cultured for five days with irradiated transfectants in vitro. Thereafter, cells were harvested and depleted of $CD4^+$ or $CD8^+$ T cells by using monoclonal anti-CD4 or anti-CD8 antibodies plus complement. Depleted populations were then tested against ^{51}Cr-EG7-*ova* in the *ova* system or ^{51}Cr-P13.1 in the β-gal system. The data shown in

FIG. 4 indicates that, in the *ova* system, depletion of CD8$^+$ T cells abrogated cytolytic activity conferred by the whole effector cell population. However, depletion of CD4$^+$ T cell population did not have any effect on the lysis of EG7-*ova*.

Similarly, in the β-gal system, depletion of CD8$^+$ T cells abrogated the cytolytic activity of β-gal-antigen formulation immunized spleen cells.

Example 4: Soluble *ova* in AF prime CD8$^+$ T cells

To demonstrate that *ova*-AF primes CD8$^+$T cell populations in vivo, and is critical for in vitro secondary response, CD4$^+$ or CD8$^+$ populations were depleted from spleens of *ova*-AF immunized mice and from naive mice. These treated populations were then stimulated *in vitro* with EG7-*ova* alone, or in a combination of CD4$^+$ and CD8$^+$ T cells from *ova*-AF immunized mice, or in various combination of CD4$^+$ or CD8$^+$ T cells from *ova*-AF immunized mice with the CD4$^+$ or CD8$^+$ cells from naive mice. FIG. 5 shows that primed CD8$^+$ cells are essential for the manifestation of a secondary CTL response *in vitro*. These data also indicate that for the effective secondary CTL response *in vitro*, CD4$^+$ T cells are required. CD4$^+$ cells are not needed for priming. Similarly, CD8$^+$ T cells were required for the manifestation of B-gal specific secondary CTL response *in vitro*.

The above examples demonstrate the effect of the antigen formulation on the induction of class I restricted CTL responses against soluble protein antigens. The antigen formulation mediated soluble antigen induced CTL priming, and is similar in activity to that induced by transfectants and by splenocytes cytoplasmically loaded with soluble *ova* or β-gal. In the ovalbumin system, EG7-*ova*, cytoplasmically loaded *ova* splenocytes, and *ova*-AF

induced: (a) class I restricted $CD8^+$ CTL; (b) CTL that recognize target sensitized with *ova* 253-276 synthetic peptide; and (c) long lived CTL after only one immunization. In the β-galactosidase system, the β-gal-AF induced CTL that recognize β-gal expressing transfectant C3-4, and also the untransfected P815 cells sensitized with alkali digested β-gal. This is analogous to what was observed with CTL induced by immunization with spleen cells cytoplasmically loaded with β-galactosidase. The induction of *ova*-specific CTL by antigen formulation is unique because neither *ova* encapsulated in a pH sensitive liposome, nor in alum, could induce CTL priming *in vivo*.

These examples indicate that the antigen formulation used above, and its equivalents, are useful in human therapy and in vaccine development for the induction of CTL in various cancers and viral diseases.

Example 5:

This is a specific example to show the use of the above AF on producing class I restricted CTL priming by soluble gp120 from HIV.

The gp160 IIIB expressing cell line (15-12) was produced in the Balb/c fibroblast-derived 3T3 cell line. It was obtained from Drs. Ron Germain and Jay Berzofsky, National Institute of Health, Bethesda, M.D. The gp160 expressing cell line was employed for in vitro stimulation of in vivo primed splenic lymphocytes, and also used as target for gp160 specific CTL induction. Balb/c mice were immunized once with 10 μg of gp160 per mouse with or without AF. Mice were injected at footpads and tailbase subcutaneously. Spleen cells were taken from the immunized mice after two weeks of immunizations and in vitro stimulated with irradiated gp160 transfectants. After five days of culture in vitro, priming was assessed by the

presence of specific effectors capable of lysing gp160 transfectants, and not the untransfected cell lines. The results are shown in Fig. 7, where CTL response is potentiated with AF and gp120.

The following example demonstrates the use of antigen formulations of this invention with use of only one or two components. These examples demonstrate that CTL-responses can be induced with only two of the above three components.

Example 6: Determination of critical components necessary for CTL induction

To determine whether all the above-noted components are necessary for antigen specific CTL induction, mice were immunized with ovalbumin in a microfluidized formulation of various combinations of two of the three components presented in the AF above. Two component combinations used were as follows; Squalane/TWEEN in PBS, Squalane/Pluronic in PBS or Pluronic/TWEEN in PBS. Another set of groups were included where mice were immunized with *ova* formulated in a one component system i.e., Squalane in PBS, Pluronic in PBS or TWEEN in PBS only. The above three component antigen formulation was modified to exclude one component at a time, constituting PBS in its place.

The above antigen formulations consist of: 0.300g TWEEN 80 (Aldrich, WI), 1.875g Pluronic L121 (BASF, NJ), and 7.5g Squalane (Aldrich, WI), brought to 50 ml with PBS.

The two-component formulations were:

Squalane/TWEEN: 0.300g TWEEN 80, and 7.5g Squalane, brought to 50 ml with PBS.

Pluronic/TWEEN: 1.875g Pluronic L121, and 0.300 g TWEEN 80, brought to 50 ml with PBS.

Pluronic/Squalane: 1.875g Pluronic L121, and 7.5g Squalane, brought to 50 ml with PBS.

The samples were then processed through a microfluidizer, model 110T, Microfluidics corp, and bottled and stored at 4°C until use.

Ovalbumin (Sigma, MO) was weighted and brought to a 0.3mg/ml solution in HBSS (Whittaker, Supra). The stock 0.3mg/ml solution was combined with the two component formulation in the following amounts: 5 parts Ovalbumin 0.3 mg/ml solution, 3.3 parts 2 component formulation, and 1.7 parts HBSS.

The formulation was vortexed and kept on ice until injected. All solutions were combined just prior to injection.

Each mouse received 200 μl of one formulation containing 30 μl of OVA by injection in both hind footpads and any remaining solution was injected subcutaneously at the tail base. Mice were allowed to rest for two to four weeks prior to spleen harvest.

Two weeks after immunizations, spleen cells were prepared and in vitro stimulated with irradiated EG7-OVA. After five days of culture, the presence of OVA specific CTL was measured by testing against ^{51}Cr-EG7-OVA or ^{51}Cr-EL4 in a 4 hour ^{51}Cr release assay. The data shown in FIGS. 8-10 demonstrate that Ovalbumin formulated in microfluidized two component system can prime OVA specific CTLs in vivo.

We further evaluated the relative contribution of the individual components for their ability to induce CTL when combined with protein antigens. For immunization purposes soluble antigen was mixed with microfluidized excipients to obtain a stable homogeneous emulsion with particle sizes ranging from 250-300 nm. To further define the components of squalane-Tween 80-pluronic (STP) formulation responsible for CTL induction, we immunized

mice with ova in squalane-Tween 80 (ST) mixture, pluronic-Tween 80 (PT) mixture or squalane-pluronic (SP) mixture and as a control, in squalane (S), Tween 80 (T) or pluronic (P). Mice were also immunized with ova-SAFm (containing 70 μg of MDP) or ova-alum as adjuvant controls. For a positive control, mice were immunized with spleen cells cytoplasmically loaded with soluble ova. Other combinations and substitutes were also used, and the results are presented in Table 1.

For the detection of CTL priming studies, mice were immunized once. Two weeks after the immunization, spleen cells were mixed with irradiated EG7-ova (the ova expressing EL4 cells) for five days and tested against ^{51}Cr-EG7-ova or ^{51}Cr-EL4 cells. The results (Fig. 11) demonstrate that 30 μg of ova in combination with STP or ST primes class I restricted CTL response in mice. The priming of ova specific CTL by ova in STP or by ova in ST appears to be better than that induced by spleen cells cytoplasmically loaded with soluble ova. Ova in PT or in SP could induce ova specific CTL responses in mice but inconsistently and poorly. Unlike SAFm, the addition of MDP to ST formulation did not compromise the ova specific CTL induction in mice (Table 2). No ova specific CTL induction occurred when mice were immunized with ova mixed with the individual components, S, P or T nor when mice were immunized with ova-SAFm or ova-alum. Mice immunized with as much as 1 mg ova in (a) HBSS, in (b) SAFm or (c) absorbed to alum did not prime ova specific CTL.

Table 2

Induction of *ova* specific CTL response is not blocked by ST + MDP

Stimulator	Target**		E-T	*ova*-ST	% cytotoxicity in mice immunized with* *ova*-ST	*ova*-ST-MDP 300 µg mouse	*ova*-ST-MDP 72µg mouse
EG7-*ova*	EG7-*ova*	100:1	0	100	82	76	
		33:1	0	86	67	62	
		11:1	0	33	39	25	
		3:1	0	6	13	3	
		1:1	0	0	0	0	
		3:1	0	0	0	0	

* mice were immunized with 30µg *ova* in various formulations

** % cytoxicity was calculated by subtracting the percent kill against antigen non-expressing cells lines

Example 7: Components Necessary for *ova* Specific Antibody Production

Mice were immunized three times at 2 week intervals with 30 µg of *ova* in HBSS, STP, ST, PT or SP. As a positive control, mice were also immunized with *ova*-SAFm, as SAFm is known to induce a strong antibody response. Seven days after the second and third immunizations, mice were bled and the sera tested for *ova* specific antibody response. The results are shown in Table 3. They indicate that mice immunized with *ova* in STP, ST or in SAFm display similar anti-*ova* responses after two immunizations.

Table 3

Induction of anti-ova antibody response

30μg ova/animal formulation	# mice responded/ # mice injected	1/dilution sera titer
HBSS	0/3	<1/20, <1/20, <1/20
STP	3/3	<1/4860, >1/4860, <1/4860
ST	3/3	>1/4860, >1/4860, >1/4860
PT	NA	NA, NA, NA
SP	NA	NA, NA, NA
SAF-M	3/3	1/4860, 1/4860, 1/4860

* N/A; not available

Example 8: HIV gp120 Specific CTL Induction

HIV gp120 IIIB was used as a second antigen system to determine CTL induction in STP, ST or in MP-T. Mice were immunized with 1 μg of gp120 IIIb in HBSS, STP, PT or in ST. As a control, mice were immunized with 1 μg of gp120IIIb in SAFm or CFA (Complete Freund's Adjuvant) or in RIBI adjuvant system containing MPL (monophoshoryl lipid A) and TDM (trehalose dimycolite). Three weeks after the immunization, spleen cells were prepared and stimulated *in vitro* with mitomycin treated transfectant cells 15-12 or with the 18IIIb peptide. After five days of culture, the resultant effector cells were tested against vaccinia:gp160 IIIB, or parental vaccinia infected P815 cells as targets. The results demonstrate that the gp120-Squalane-TWEEN 80 formulation and not gp120-Squalane-TWEEN 80 pluronic

formulation or gp120-HBSS induced gp120 specific CTL response in mice (Table 4).

Table 4

Induction of gp120 specific CTL response in mice

Stimulator	Target**	E-T	% cytotoxicity in mice immunized with* gp120-HBSS	gp120-ST	gp120-STP
18IIIb/IL2	vac:gp120	100:1	23	42	NA***
		33:1	23	38	NA
		11:1	0	0	NA
		3:1	0	35	NA
18IIIb/IL2	15-12	100:1	0	50	0
		33:1	0	35	0
		11:1	0	27	0
		3.1	0	18	0
18IIIb/IL2	3T3+18IIIb	100:1	0	59	13
		33:1	0	59	2
		11:1	0	57	0
		3:1	0	29	0
15-12	vac:gp120	100:1	35	84	NA
		33:1	19	65	NA
		11:1	12	37	NA
		3:1	0	22	NA
		1:1	0	0	NA

* mice were immunized with 1 μg of gp120III in various formulations

** % cytotoxicity was calculated by subtracting the percent kill against antigen non-expressing cell lines

*** NA; not available

Example 9: Induction of gp120 Specific Humoral Response in Mice

For the induction of gp120 specific humoral responses, mice were immunized with 1 μg of gp120IIIb three times at two-week intervals. The animals were bled and tested for the presence of IgG antibodies detecting gp120IIIb in a solid phase ELISA assay. The results demonstrate that gp120-ST is a better immunogen than gp120-HBSS, gp120SAFm (Table 5), or gp120-STP.

Table 5

Induction of anti-gp120 antibody response

1μg gp120/animal formulation	# mice responded/ # mice injected	1/dilution sera titer
HBSS	0/3	<1/20, <1/20, <1/20
STP	1/3	<1/20, >1/4860, <1/20
ST	3/3	>1/4860, >1/4860, >1/4860
PT	3/3	>1/4860, >1/4860, >1/4860
SP	2/3	<1/20, 1/540, 1/540
Saf-M	2/3	1/180, >1/4860, 1/540

Example 10: gp120 Specific antibody Responses in monkeys

Monkeys (two per group) were immunized with gp120-SAFm, gp120-SPT, gp120-ST, or gp120-HBSS. As a control, a group of monkeys were immunized with recombinant vaccinia containing gp160 IIIb. Monkeys were immunized at two week intervals and bled two weeks and three weeks after the second immunization. Pre- and immune sera from each monkey was serially diluted and assayed for anti-gp120 activity in an ELISA as described in the materials and methods. The data (Figure 12) indicate that monkeys immunized with gp120-STP or gp120-SAFm induced similar responses in monkeys. One monkey immunized with gp120-ST, induced anti-gp120 response similar to the gp120-SAFm or gp120-SPT immunized group. One monkey immunized with gp120-ST did not induce a strong anti-gp120 response after two immunizations.

Example 11: In vivo activity of AF in combination with HPV 16 E7

1. Generation of recombinant HPV 16 E7 Protein for Immunization

a) PCR and cloning of the E7 gene

The HPV 16 E7 gene was cloned from a plasmid obtained from Dr. Karen Vousden (Ludwig Institute) encoding the E7 gene derived from the carcinoma cell line CaSki. The coding regions were amplified by PCR using primers that encode the 5′ and 3′ ends of the genes flanked by Bam HI and Sal I cloning sites. The E7 PCR product was ligated into the pGEX - 4T-1 expression vector (Pharmacia Biotech) resulting in the pGEX.E7 expression plasmid. *E. coli* strain XL1 - blue (stratagene) was transfected with the pGEX.E7 expression plasmid. The sequence of the E7 was

obtained from the plasmids of the resulting colonies and was identical to the E7 sequence obtained from CaSki cells.

b) Production of purification of bacterially-expressed E7

The pGEX.E7 bacterial expression plasmid encodes a glutathione-S-transferase (GST) fusion protein consisting of the GST at the amino-terminus, a thrombin protease cleavage site and the E7 protein at the carboxy-terminus. E7 protein was produced and purified as described in the product information literature from the manufacturer of the pGEX-4T-1 vector (Pharmacia Biotech). Briefly, bacteria containing the pGEX.E7 expression plasmid was induced to express the fusion protein by the addition of isopropyl b-D-thiogalactosidase to the culture medium. The cells were harvested and lysed by mild sonication. The lysate was applied to Glutathione Sepharose 4B (Pharmacia Biotech). After the fusion protein bound to the matrix, the resin was washed to remove non-specifically bound proteins. The bound fusion protein was digested with thrombin to release the E7 protein from the GST fusion partner.

The E7 protein preparation was analyzed by SDS-PAGE and the E7 protein concentration was determined by Bradford analysis (BioRad). 9 mg soluble E7 protein was obtained per liter of bacterial culture.

2. Generation of the X21 E7 Transfectant

Coding sequences for the HPV16 E7 protein (see above) have been inserted into the IDEC proprietary eukaryotic expression plasmid INPEP4. Within this vector, E7 expression is controlled by the Cytomegalovirus promoter/enhancer transcriptional elements. In addition, the first three nucleotides of the E7 coding sequence have

been removed and replaced with an immunoglobulin light chain leader sequence placed immediately upstream and in frame with the E7 coding region. Following transfection into the mouse cell line X21 individual G418 resistant clones were examined by northern blot analyses for E7 message production. Every clone displayed detectable E7 message. Western blot analysis of cell lysates from the two of those clones, 4E7 and 1C7, (HOPE1 and HOPE2 respectively) were then performed and demonstrated E7 protein production.

3. *In vivo* Activity of E7/AF Soluble Antigen Immunization

Female mice of C3H background ($H2^{k/k}$, Harlan Sprague Dawley) were used in these studies. Animals were maintained according to "Guide for the Care and Use of Laboratory Animals" (DHHS Publication No. NIH 86-23, Bethesda, MD:NIH, 1985), and received food and water *ad libitum*. The E7 transfectant cell line HOPE2 $H2^{k/k}$) was used in these studies. The tumor cell line was maintained by serial passage *in vitro*.

This cell line has been shown to maintain E7 cytoplasmic antigen expression, as detected by western blot analysis, following repeated *in vitro* passages. Tumors were initiated in syngeneic C3H mice by subcutaneous injection of 150,000 *in vitro* passaged cells.

Tumors were measured in 2 perpendicular directions at biweekly intervals. Tumor volume (V) was calculated according to the following formula:

$$V\ (mm^3) = (L x W^2) \text{ divided by } 2$$

where:
L = longest axis measurement in mm
W = perpendicular axis (mm)

Data in Table 6 are presented as tumor Mice (number of tumor bearing animals over the total number of animals injected). Data in Figures 13 and 14 are presented as median tumor size (mm^3) of each treatment or control group. Each treatment group was compared to a control group that did not receive therapy. Therapy began 10 days after incoculation of HOPE2 cells, when a majority of the tumors were palpable (approx. 50-75 mm^3). Therapy was initiated by immunization of mice with soluble E7 protein in AF or Alum adjuvants (subcutaneously in a total volume of 0.2 ml). Directly before immunization, AF was mixed for 60 seconds with E7 protein in Hanks Balanced Salt Solution (HBSS) such that each mouse received either 30 ug or 90 ug E7 protein 0.2 ml. Alum (Pierce Chemical Co.) was mixed with E7 protein, according to instructions by the manufacture, such that each animal received 90 ug E7 protein in 0.2 ml per mouse. Animals in a second treatment group received a second immunization 9 days later (19 days after tumor cell inoculation). Booster Immunization were prepared immediately before inoculation, as described above.

In this example (Table 6: Xp #233), 41 days after tumor cell inoculation only 4/8 and 5/8 of mice receiving a single injection of soluble E7 in aF (30 ug or 90 ug respectively) had measurable tumors. In contrast, all of the mice immunized with E7 protein in Alum (8/8) had actively growing tumors. Additionally, as shown in Figure 13, significant inhibition of tumor growth was observed only in treatment groups immunized with E7 protein in aF as compared to control (untreated) or Alum treatment groups. Inhibition of tumor growth (Figure 13) or increased tumor regression rates (Table 6 was not observed in mice that received a single injection of E7 in Alum.

Similar results were also observed using treatment groups that received two immunizations at days 10 and 19 after tumor challenge (Table 6 and Figure 14), although some tumor growth retardation was observed with mice receiving two injections of E7 in Alum.

The results indicate that significant antitumor activity as measured by a decreased number of tumor bearing mice and inhibition of tumor growth was observed following immunization of soluble E7 in AF. In contrast, all animals immunized with either a single or double injection of soluble E7 protein in Alum had growing tumors. In summary, immunization with soluble E7 protein in AF resulted in a significant inhibition of tumor cell growth that was not observed using soluble E7 immunization in Alum.

Table 6: Antitumor activity of soluble E7 immunization in adjuvant

Exp. #	Treatment	Dose (ug/mouse)	Tumor Animals[a] Day 41
223	Control	-	7/8
223	E7 in AF	30 ug x 1[b]	4/8
223	E7 in AF	90 ug x 1	5/8
223	E7 in Alum	90 ug x 1	8/8
223	E7 in AF	30 ug x 2[c]	3/8
223	E7 in AF	90 ug x 2	1/4
223	E7 in Alum	90 ug x 2	8/8

a. Number of tumor bearing mice/total number inoculated
b. All immunizations started on Day 10 post implant
c. Second immunication (x2) on Day 19 post implant

Other embodiments are within the following claims.

CLAIMS:

1. A composition comprising an antigen mixed with a microfluidized antigen formulation comprising:

(a) a stabilizing detergent,

(b) a micelle-forming agent, and

(c) a biodegradable and biocompatible oil,

said antigen formulation being formulated as a stable oil-in-water emulsion, said antigen formulation being substantially free of immunostimulating peptides and wherein said composition upon administration to an animal selected from the group consisting of humans, domesticated animals and agricultural animals is capable of inducing a specific cytotoxic T-lymphocyte response against the antigen contained in the composition.

2. The composition of claim 1, wherein said antigen is chosen from antigenic portions of the HIV antigens: gp160, gag, pol, Nef, Tat, and Rev; the malaria antigens: CS protein and Sporozoite surface protein 2; the Hepatitis B surface antigens: Pre-S1, Pre-S2, HBc Ag, and HBe Ag; the influenza antigens: HA, NP and NA; Hepatitis A surface antigens; the Herpes virus antigens: EBV gp340, EBV gp85, HSV gB, HSV gD, HSV gH, HSV early protein product, cytomegalovirus gB, cytomegalovirus gH, and IE protein gp72; the respiratory syncytial virus antigens: F protein, G protein, and N protein; and the tumor antigens: carcinoma CEA, carcinoma associated mucin, carcinoma P21, carcinoma P53, melanoma MPG, melanoma p97, carcinoma Neu oncogene product, carcinoma p53 gene product, and mutated p21 ras protein.

3. A composition comprising an antigen mixed with a microfluidized antigen formulation comprising:

(a) a stabilizing detergent,

(b) a micelle-forming agent, and

(c) a biodegradable and biocompatible oil,

said antigen formulation being formulated as a stable oil-in-water emulsion, said antigen formulation lacking immunostimulating peptides and wherein said composition upon administration to an animal selected from the group consisting of humans, domesticated animals and agricultural animals is capable of inducing a specific cytotoxic T-lymphocyte response against the antigen contained in the composition.

4. The composition of claim 3, wherein said antigen formulation consists essentially of said detergent, agent, and oil.

5. The composition of claim 3, wherein said antigen formulation is non-toxic to said human or domesticated or agricultural animal.

6. The composition of claim 3, wherein said antigen is chosen from the HIV antigens: gp160, gag, pol, Nef, Tat, and Rev; the malaria antigens: CS protein and Sporozoite surface protein 2; the Hepatitis B surface antigens: Pre-S1, Pre-S2, HBc Ag, and HBe Ag; the influenza antigens: HA, NP and NA; Hepatitis A surface antigens; the Herpes virus antigens: EBV gp340, EBV gp85, HSV gB, HSV gD, HSV gH, HSV early protein product, cytomegalovirus gB, cytomegalovirus gH, and IE protein gP72; the respiratory syncytial virus antigens: F protein, G protein, and N - protein; and the tumor antigens: carcinoma CEA, carcinoma associated mucin, carcinoma P21,

carcinoma P53, melanoma MPG, melanoma p97, carcinoma Neu oncogene product, carcinoma p53 gene product, a human papillomavirus antigen, the prostate specific antigen (PSA) and mutated p21 ras protein.

7. A method for inducing a cytotoxic T-lymphocyte response in an animal selected from the group consisting of humans, domesticated animals and agricultural animals, comprising:

administering to said animal an admixture comprising an antigen and a microfluidized antigen formulation, said antigen formulation comprising:

(a) a stabilizing detergent,

(b) a micelle-forming agent, and

(c) a biodegradable and biocompatible oil,

said antigen formulation lacking an immunostimulating peptide component, said antigen formulation being formulated as a stable oil-in-water emulsion; wherein said admixture is administered to said animal in an amount sufficient to induce a cytotoxic T-lymphocyte response in said animal which is specific for the antigen contained in said admixture.

8. The method of claim 7, wherein said antigen formulation consists essentially of said detergent, agent, and oil.

9. The method of claim 7, wherein said method consists essentially of a single administration of said mixture to said human or said animal.

10. The method of claim 7, wherein said human or said animal is infected with a virus or suffers one or more symptoms of infection from said virus.

11. The method of claim 7, wherein said antigen formulation is non-toxic to said human or said animal.

12. The method of claim 7, wherein said antigen is chose from the HIV antigens: gp160, gag, pol, Nef, Tat, and Rev; the malaria antigens: CS protein and Sporozoite surface protein 2; the Hepatitis B surface antigens: Pre-S1, Pre-S2, HBc Ag, and HBe Ag; the influenza antigens: HA, NP and HA; Hepatitis A surface antigens; the Herpes virus antigens: EBV gp340, EBV gp85, HSV gB, HSV gD, HSV gH, HSV early protein product, cytomegalovirus gB, cytomegalovirus gH, and IE protein gP72; the respiratory syncytial virus antigens: F protein, G protein, and N protein; and the tumor antigens: carcinoma CEA, carcinoma associated mucin, carcinoma P21, carcinoma P53, melanoma MPG, melanoma p97, and carcinoma Neu oncogene product, carcinoma p53 gene product, and mutated p21 ras protein.

13. A method of treating a patient infected with HIV virus, comprising administering a composition comprising an HIV antigen mixed with a microfluidized antigen formulation comprising:

(a) a stabilizing detergent,

(b) a micelle-forming agent, and

(c) a biodegradable and biocompatible oil,

said antigen formulation lacking an immunostimulating peptide component, and being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient.

14. The method of claim 13, wherein said HIV antigen is selected from gp160, gag, pol, Nef, Tat, and Rev.

15. A method of treating a patient suffering from malaria, comprising administering a microfluidized composition comprising a malaria-associated antigen mixed with an antigen formulation comprising:

(a) a stabilizing detergent,

(b) a micelle-forming agent, and

c) a biodegradable and biocompatible oil,

said antigen formulation lacking an immunostimulating peptide component, and being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient.

16. The method of claim 15, wherein said malaria-associated antigen is selected from CS protein, and Sporozoite surface protein 2.

17. A method of treating a patient suffering from influenza, comprising administering a composition comprising an influenza-associated antigen mixed with a microfluidized antigen formulation comprising:

(a) a stabilizing detergent,

(b) a micelle-forming agent, and

(c) a biodegradable and biocompatible oil,

said antigen formulation lacking an immunostimulating peptide component, and being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient.

18. The method of claim 17, wherein said influenza-associated antigen is selected from HA, NP, and NA.

19. A method of treating a patient suffering from hepatitis, comprising administering a composition comprising a hepatitis-associated antigen mixed with a microfluidized antigen formulation comprising:

(a) a stabilizing detergent,

(b) a micelle-forming agent, and

(c) a biodegradable and biocompatible oil,

said antigen formulation lacking an immunostimulating peptide component, and being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient.

20. The method of claim 19, wherein said hepatitis-associated antigen is selected from hepatitis A surface antigen, Pre-S1, Pre-S2, HBc Ag, and HBe Ag.

21. A method of treating a patient suffering from a cancer, comprising administering a composition comprising a cancer-associated antigen mixed with a microfluidized antigen formulation comprising:

(a) a stabilizing detergent,

(b) a micelle-forming agent, and

(c) a biodegradable and biocompatible oil,

said antigen formulation lacking an immunostimulating peptide component, and being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient-to induce a cytotoxic T-lymphocyte response in said patient.

22. A method of claim 21, wherein said cancer-associated antigen is selected from Carcinoma CEA, Carcinoma associated mucin, P21, carcinoma P53, melanoma

MPG, melanoma p97, and carcinoma Neu oncogene product, carcinoma p53 gene product, and mutated p21 ras protein.

23. A method of treating a patient infected with herpes virus, comprising administering a composition comprising a herpes antigen mixed with a microfluidized antigen formulation comprising:

(a) a stabilizing detergent,

(b) a micelle-forming agent, and

(c) a biodegradable and biocompatible oil,

said antigen formulation lacking an immunostimulating peptide component, and being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient.

24. The method of claim 23, wherein said herpes virus antigen is selected from EBV gp340, EBV gp85, HSV gB, HSV gD, HSV gH, HSV early protein product, cytomegalovirus gB, cytomegalovirus gH and IE protein gP72.

25. A method of treating a patient infected with respiratory syncytial virus, comprising administering a composition comprising a respiratory syncytial antigen mixed with a microfluidized antigen formulation comprising:

(a) a stabilizing detergent,

(b) a micelle-forming agent, and

(c) a biodegradable and biocompatible oil,

said antigen formulation lacking an immunostimulating peptide component, and being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient.

26. The method of claim 25 wherein said Respiratory Syncytial virus antigen is selected from F protein, G protein, and N protein.

27. A method for inducing a cytotoxic T-lymphocyte response in a human or domesticated or agricultural animal, comprising the steps of:

administering a mixture of an antigen mixed with a microfluidized antigen formulation consisting essentially of two of:

(a) a stabilizing detergent,

(b) a micelle-forming agent, and

(c) a biodegradable and biocompatible oil,

said antigen formulation being formulated as a stable oil-in-water emulsion;

wherein said mixture is administered to said human or animal in an amount sufficient to induce a cytotoxic T-lymphocyte response in said human or animal.

28. The method of claim 27, wherein said human or domesticated or agricultural animal is infected with a virus and suffers one or more symptoms of infection from said virus.

29. The method of claim 27, wherein said antigen formulation is non-toxic to said human or domesticated or agricultural animal.

30. The method of claim 27, wherein said antigen is chosen from antigenic portions of the HIV antigens: gp160, gag, pol, Nef, Tat, and Rev; the malaria antigens: CS protein and Sporozoite surface protein 2; the Hepatitis B surface antigens: Pre-S1, Pre-S2, HBc Ag, and HBe Ag; the influenza antigens: HA, NP and NA; Hepatitis A surface

antigens; the Herpes virus antigens: EBV gp340, EBV gp85, HSV gB, HSV gD, HSV gH, HSV early protein product, cytomegalovirus gB, cytomegalovirus gH, and IE protein gp72; the respiratory syncytial virus antigens: F protein, G protein, and N protein; and the tumor antigens carcinoma CEA, carcinoma associated mucin, carcinoma P21, carcinoma P53, melanoma MPG, melanoma p97, and carcinoma Neu oncogene product, carcinoma p53 gene product, and mutated p21 ras protein.

31. A method of treating a patient infected with HIV virus, comprising administering a composition comprising an HIV antigen mixed with a microfluidized antigen formulation consisting essentially of two of:

(a) a stabilizing detergent,
(b) a micelle-forming agent, and
(c) a biodegradable and biocompatible oil;

said antigen formulation being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient.

32. The method of claim 31, wherein said HIV antigen is selected from gp160, gag, pol, Nef, Tat, and Rv.

33. A method of treating a patient suffering from malaria, comprising administering a composition comprising a malaria-associated antigen mixed with a microfluidized antigen formulation consisting essentially of two of:

(a) a stabilizing detergent,
(b) a micelle-forming agent, and
(c) a biodegradable and biocompatible oil,

said antigen formulation being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient.

34. The method of claim 33, wherein said malaria-associated antigen is selected from CS protein, and Sporozoite surface protein 2.

35. A method of treating a patient suffering from influenza, comprising administering a composition comprising an influenza-associated antigen mixed with a microfluidized antigen formulation consisting essentially of two of:

(a) a stabilizing detergent,
(b) a micelle-forming agent, and
(c) a biodegradable and biocompatible oil,

said antigen formulation being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient.

36. The method of claim 35, wherein said influenza-associated antigen is selected from HA, NP, and NA.

37. A method of treating a patient suffering from hepatitis, comprising administering a composition comprising a hepatitis-associated antigen mixed with a microfluidized antigen formulation consisting essentially of two of:

(a) a stabilizing detergent,
(b) a micelle-forming agent, and
(c) a biodegradable and biocompatible oil,

said antigen formulation being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient.

38. The method of claim 37, wherein said hepatitis-associated antigen is selected from hepatitis A surface antigen, Pre-S1, Pre-S2, HBc Ag, and HBe Ag.

39. A method of treating a patient suffering from a cancer, comprising administering a composition comprising a cancer-associated antigen mixed with a microfluidized antigen formulation consisting essentially of two of:

(a) a stabilizing detergent,
(b) a micelle-forming agent, and
(c) a biodegradable and biocompatible oil,

said antigen formulation being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient.

40. The method of claim 39, wherein said cancer-associated antigen is selected from Carcinoma CEA, Carcinoma associated mucin, P21, carcinoma P53, melanoma MPG, melanoma p97, and carcinoma Neu oncogene product, carcinoma p53 gene product, and mutated p21 ras protein.

41. A method of treating a patient infected with herpes virus, comprising administering a composition comprising a herpes antigen mixed with a microfluidized antigen formulation consisting essentially of two of:

(a) a stabilizing detergent,
(b) a micelle-forming agent, and

(c) a biodegradable and biocompatible oil,

said antigen formulation being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient.

42. The method of claim 41, wherein said herpes virus antigen is selected from EBV gp340, EBV gp85, HSV gB, HSV gD, HSV gH, HSV early protein product, cytomegalovirus gB, cytomegalovirus gH and IE protein gP72.

43. A method of treating a patient infected with respiratory syncytial virus, comprising administering a respiratory syncytial antigen mixed with a microfluidized antigen formulation consisting essentially of two of:

(a) a stabilizing detergent,

(b) a micelle-forming agent, and

(c) a biodegradable and biocompatible oil,

said antigen formulation being formulated as a stable oil-in-water emulsion; wherein said composition is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient.

44. The method of claim 43 wherein said Respiratory Syncytial virus antigen is selected from F protein, G protein, and N protein.

45. The method of any of claims 25-44 wherein said antigen formulation consists essentially of said detergent and said micelle-forming agent.

46. The method of any of claims 25-44 wherein said antigen formulation consists essentially of said detergent and said oil.

47. The method of any of claims 25-44 wherein said antigen formulation consists essentially of said oil and said micelle-forming agent.

48. The composition of claim 6 wherein said papillomavirus antigen is selected from the group consisting of the HPV16 E6 antigen, HPV16 E7 antigen, HPV18 E6 antigen, HPV18 E7 antigen, HPV6 E4 antigen, HPV6 L1 antigen, HPV11 E4 antigen and HPV11 L1 antigen.

49. A method for treating cervical cancer comprising administering an effective amount of a human papillomavirus antigen formulation according to claim 48.

50. A method for treating condyloma acuminata comprising administering an effective amount of a papillomavirus antigen formulation according to claim 48.

51. A method for treating prostate cancer comprising administering a composition according to claim 6 wherein the antigen is the prostate specific antigen.

52. The composition of claim 1 wherein the stabilizing detergent is selected from the group consisting of polysorbate 80, Tween 20, Tween 40, Tween 60, Zwittergent 3-12, Teepol HB7 and Span 85.

53. The composition of claim 1 wherein said detergent is provided in an amount ranging from approximately 0.05 to 0.5%.

54. The composition of claim 53 wherein said amount of detergent is about 0.2%.

55. The composition of claim 1 wherein said micelle-forming agent comprises a hydrophile-lipophile balance of between 0 and 2.

56. The composition of claim 1 wherein said micelle-forming agent is selected from the group consisting of poloxamer 401, Pluronic L62LF, Pluronic L101, Pluronic L64, PEG1000, Tetronic 1501, Tetronic 150R1, Tetronic 701, Tetronic 901, Tetronic 1301, and Tetronic 130R1.

57. The composition of claim 1 wherein the amount of said micelle-forming agent ranges from between 0.5 to 10%.

58. The composition of claim 57 wherein the amount of said micelle-forming agent ranges from between 1.25 and 5%.

59. The composition of claim 1 wherein the oil exhibits a melting temperature less than 60°C.

60. The composition of claim 59 wherein the oil is selected from the group consisting of squalene, squalane, eicosane, tetratetracontane, pristane, glycerol, and vegetable oils.

61. The composition of claim 1 wherein the amount of the oil ranges from between 1 and 10%.

62. The composition of claim 61 wherein the amount of the oil ranges from between 2.5 and 5%.

63. The composition of claim 1 which comprises less than 20 micrograms of muramyl dipeptide.

64. The composition of claim 63 does not comprise any muramyl dipeptide.

65. The composition of claim 1 wherein the detergent is polysorbate 80, and the micelle-forming agent is poloxamer 401.

66. The composition of claim 65 wherein the oil is squalane.

67. The composition of claim 1 wherein the detergent is selected from the group consisting of Tween 20, Tween 40 and Tween 80; the oil is selected from the group consisting of squalane, eicosane, and pristane and the micelle-forming agent is selected from the group consisting of Pluronic L62LF and polyoxamer 401.

68. The method of claim 7 wherein the detergent is selected from the group consisting of polysorbate 80, Tween 20, Tween 40, Tween 60, Zwittergent 3-12, Teepol HB7 and Span 85.

69. The method of claim 7 wherein said detergent is provided in an amount ranging from approximately 0.05 to 0.5%.

70. The method of claim 69 wherein the amount of detergent is about 0.2%.

71. The method of claim 7 wherein said micelle-forming agent comprise a hydrophile-lipophile balance of between 0 and 2.

72. The method of claim 7 wherein said micelle-forming agent is selected from the group consisting polyoxamer 401, Pluronic L62LF, Pluronic L101, Pluronic L64, PEG1000, Tetronic 1501, Tetronic 150R1, Tetronic 701, Tetronic 901, Tetronic 1301 and Tetronic 130R1.

73. The method of claim 7 wherein the amount of said micelle-forming agent ranges from between 0.5 to 10%.

74. The method of claim 71 wherein the amount of said micelle-forming agent ranges from between 1.25 and 5%.

75. The method of claim 7 wherein the oil exhibits a melting temperature of less than 60°C.

76. The method of claim 7 wherein the oil is selected from the group consisting of squalene, eicosane, tetratetracontane, glycerol, pristane, and vegetable oils.

77. The method of claim 7 wherein the amount of oil ranges from between 1 and 10%.

78. The method of claim 77 wherein the amount of oil ranges from between 2.5 and 5%.

79. The method of claim 7 wherein the admixture comprises less than 20 micrograms of muramyl dipeptide.

80. The method of clam 7 wherein the admixture does not contain any muramyl dipeptides.

81. The method of claim 7 wherein the detergent is polysorbate 80 and the micelle-forming agent is poloxamer 401.

82. The method of claim 81 wherein the oil is squalane.

83. The method of claim 7 wherein the detergent is selected from the group consisting of Tween 20, Tween 40 and Tween 80, the oil is selected from the group consisting of squalane, eicosane, olive oil and pristane and the micelle-forming agent is selected from the group consisting of polyoxamer 401, and Pluronic L62LF.

84. The method of claim 7 wherein the particle sizes in the admixture range from 250 to 300 nm.

85. The composition of claim 1 wherein the particle sizes in the composition range from 250 to 300 nm.

FIG. 1A

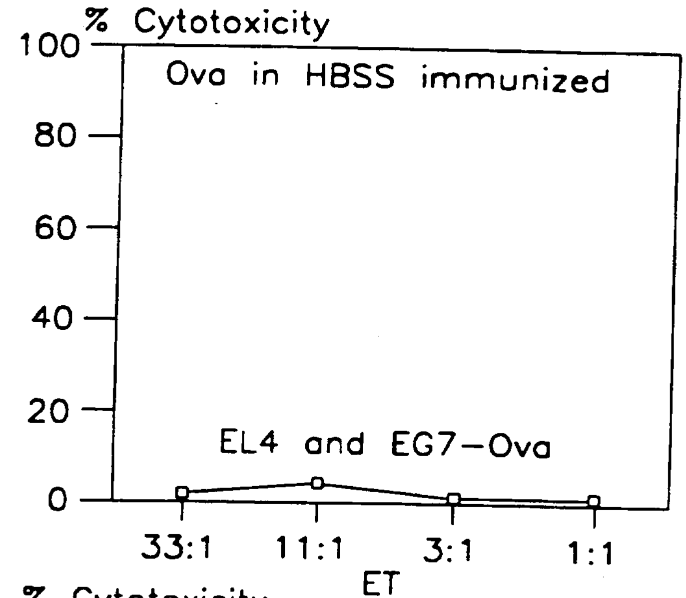


FIG. 1B

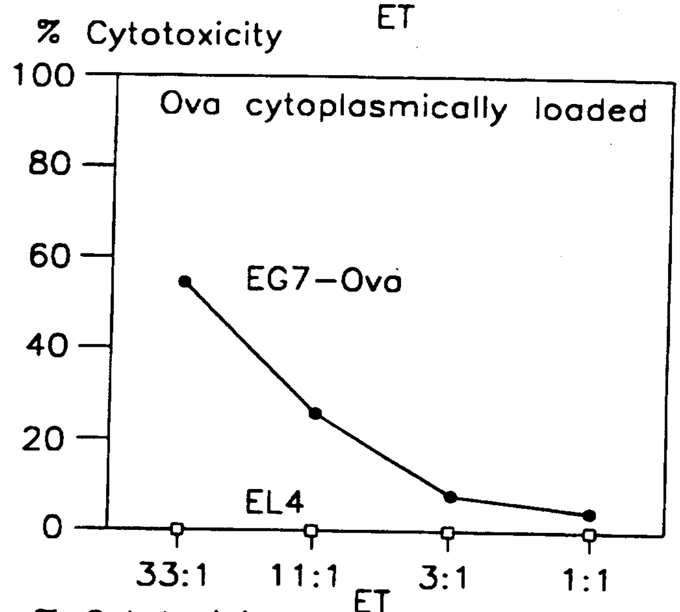
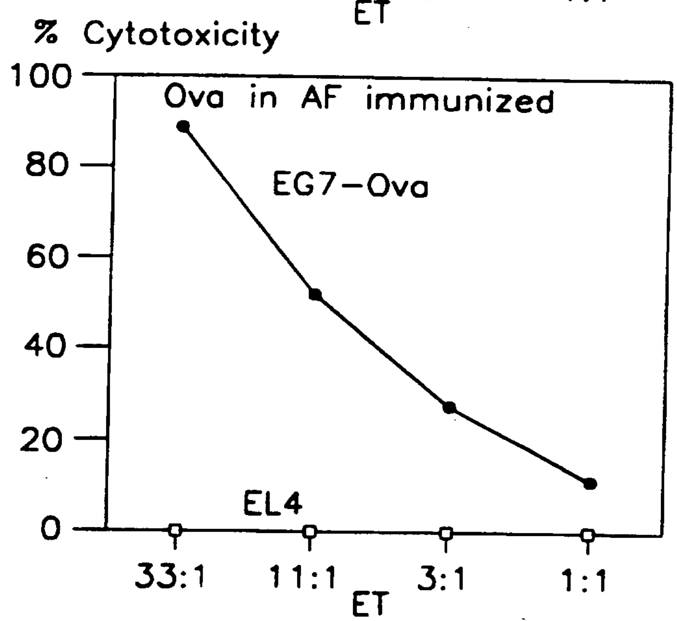


FIG. 1C



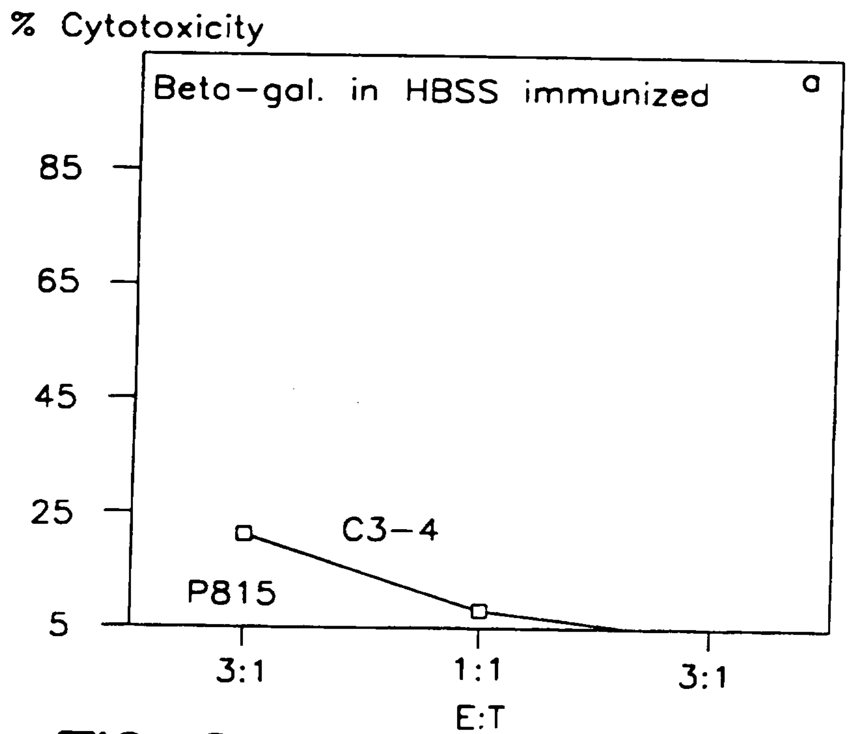

% Cytotoxicity
Beta-gal. in HBSS immunized
a
85
65
45
25
5
C3-4
P815
3:1
1:1
3:1
E:T

FIG. 2a

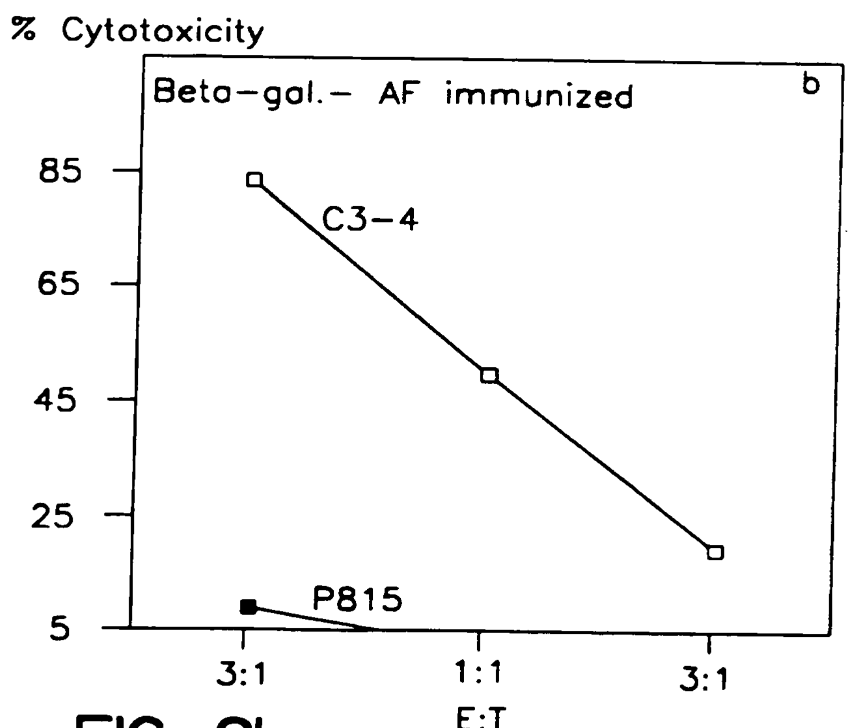

% Cytotoxicity
Beta-gal.- AF immunized
b
85
65
45
25
5
C3-4
P815
3:1
1:1
3:1
E:T

FIG. 2b

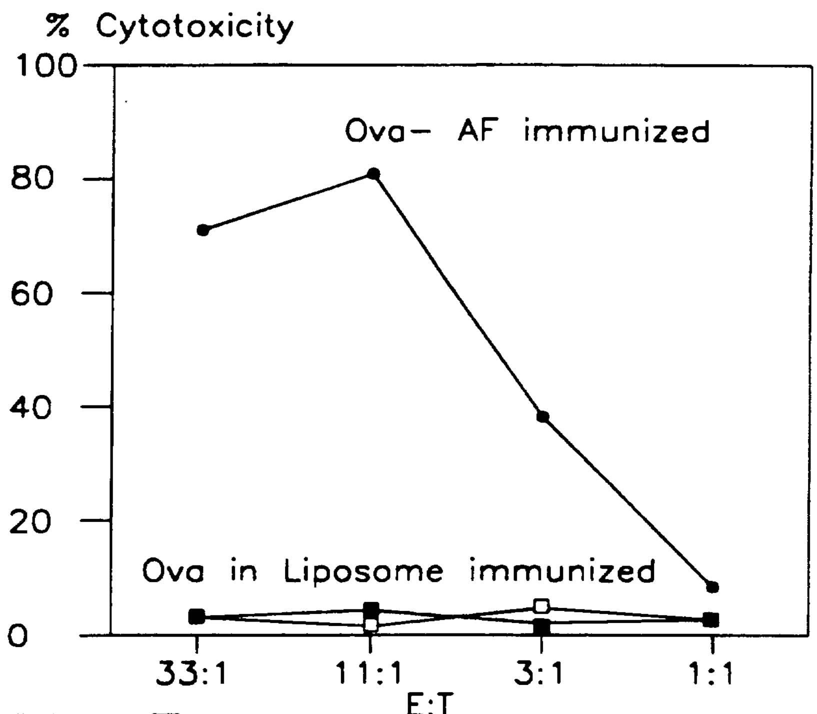

% Cytotoxicity
100
80
60
40
20
0
Ova– AF immunized
Ova in Liposome immunized
33:1
11:1
3:1
1:1
E:T

FIG. 3

FIG. 4a

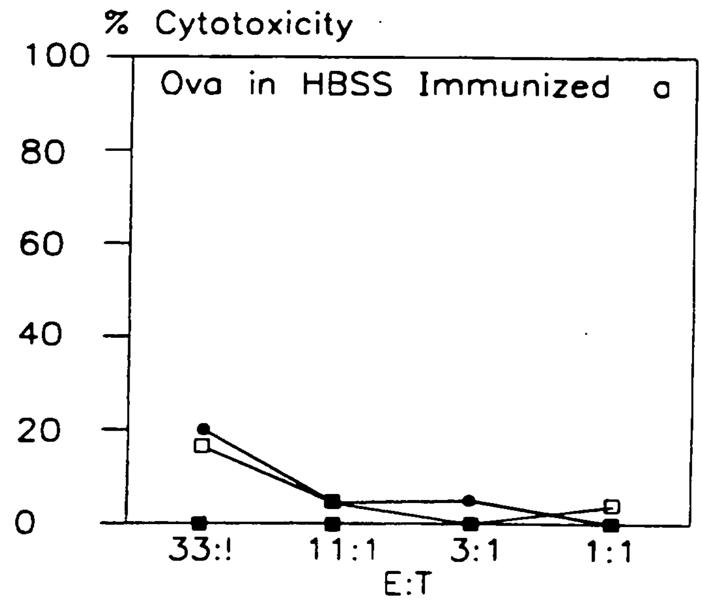


FIG. 4b

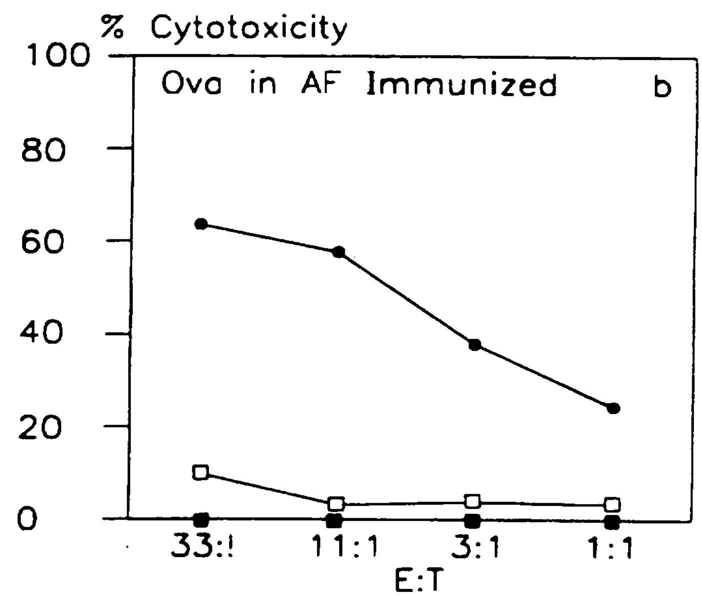
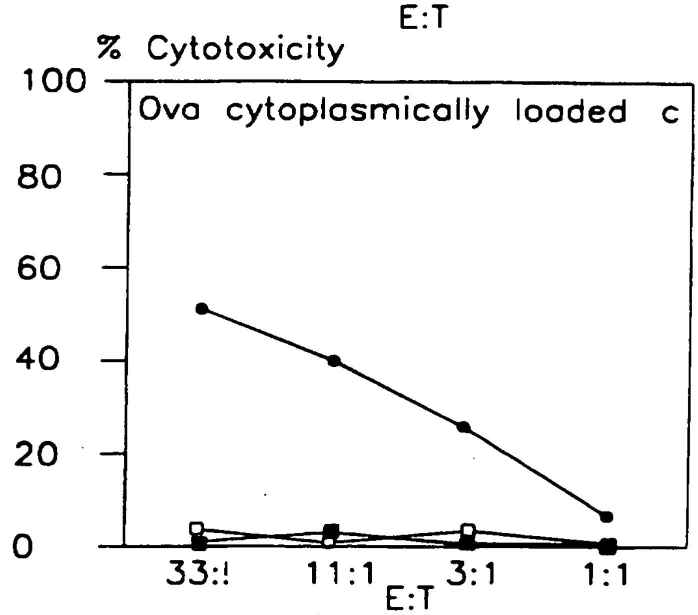


FIG. 4c



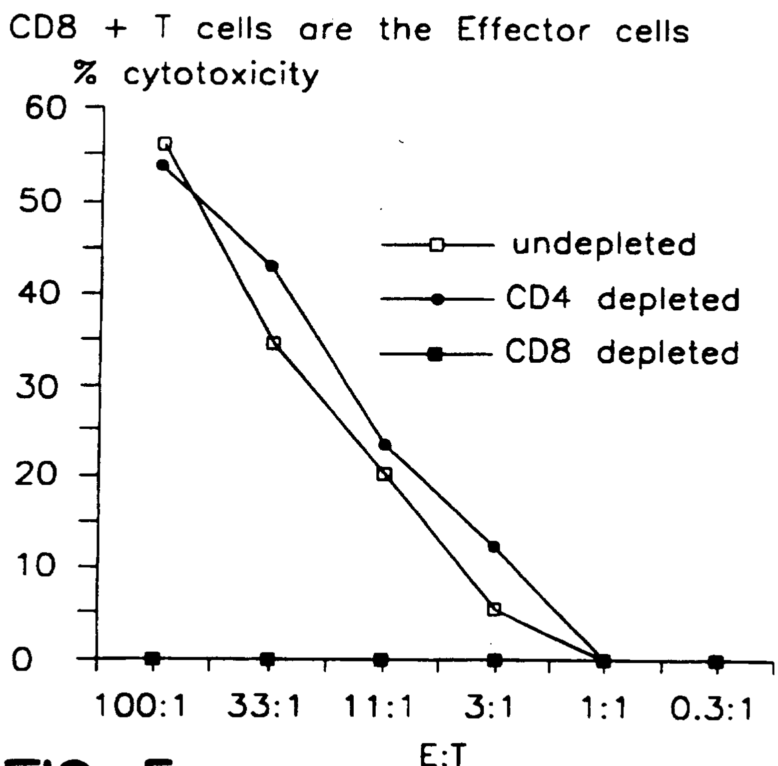

CD8 + T cells are the Effector cells
% cytotoxicity
60
50
40
30
20
10
0
undepleted
CD4 depleted
CD8 depleted
100:1
33:1
11:1
3:1
1:1
0.3:1
E:T

FIG. 5

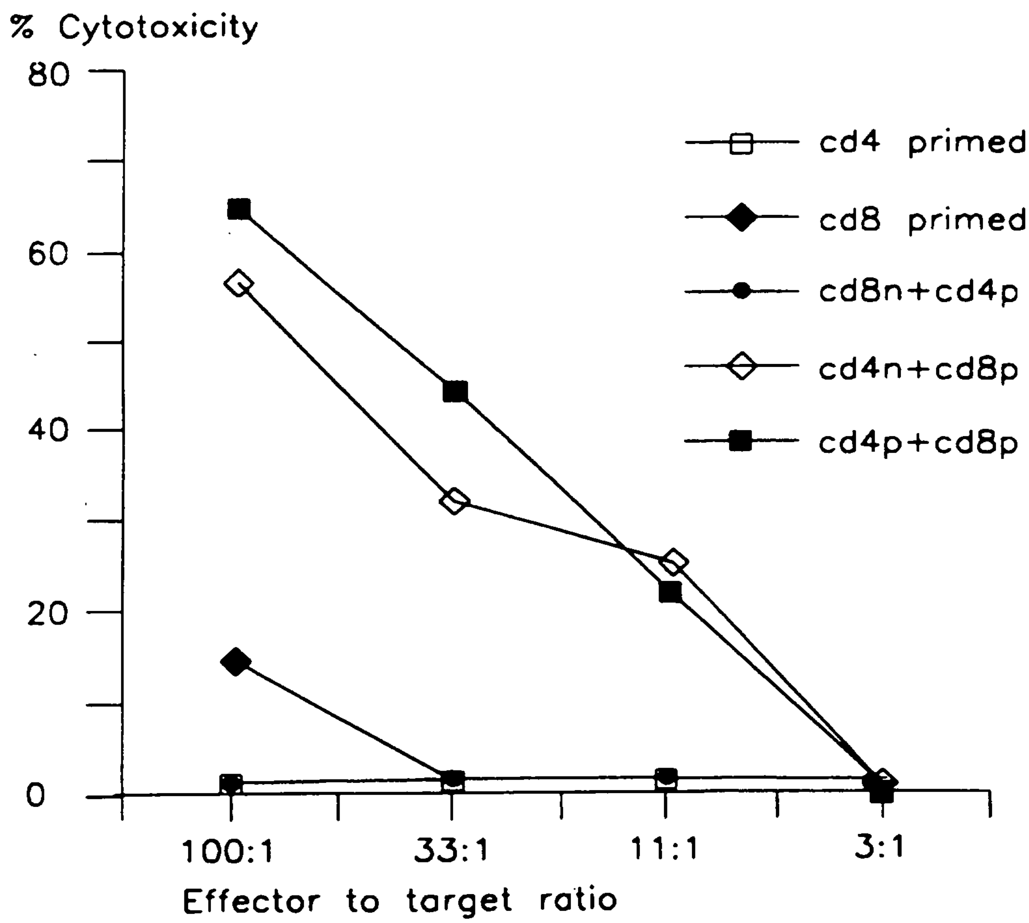

% Cytotoxicity
80
60
40
20
0
cd4 primed
cd8 primed
cd8n+cd4p
cd4n+cd8p
cd4p+cd8p
100:1
33:1
11:1
3:1
Effector to target ratio

FIG. 6

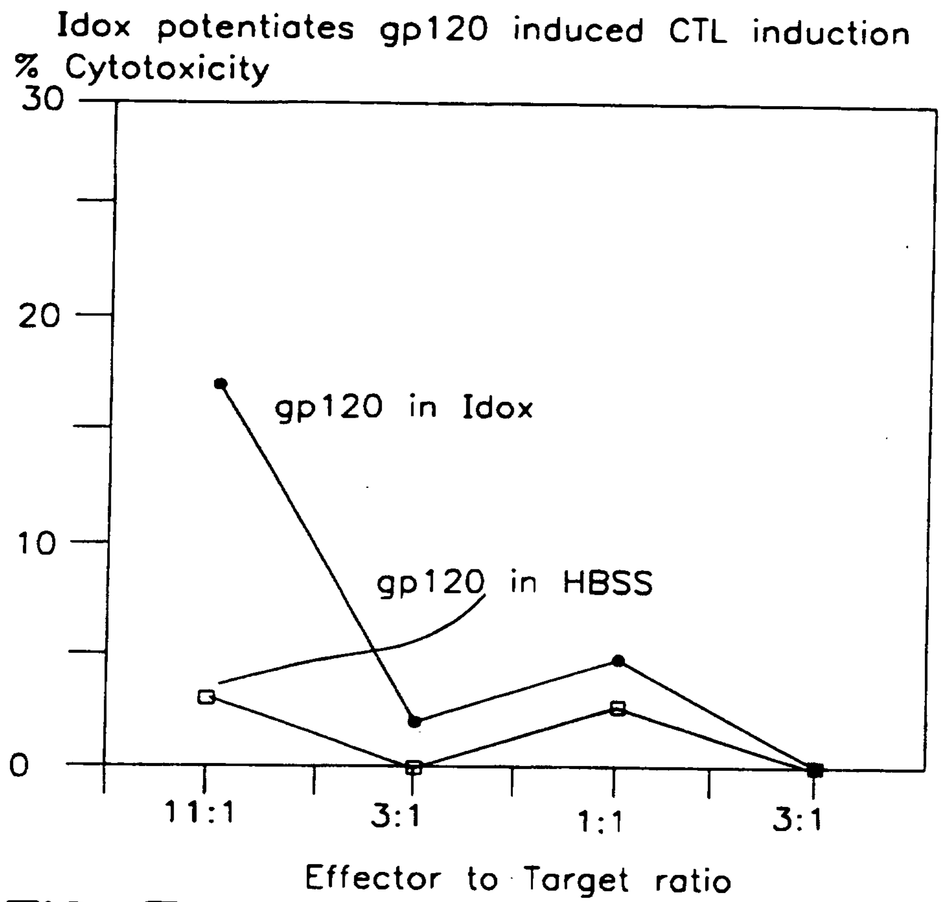
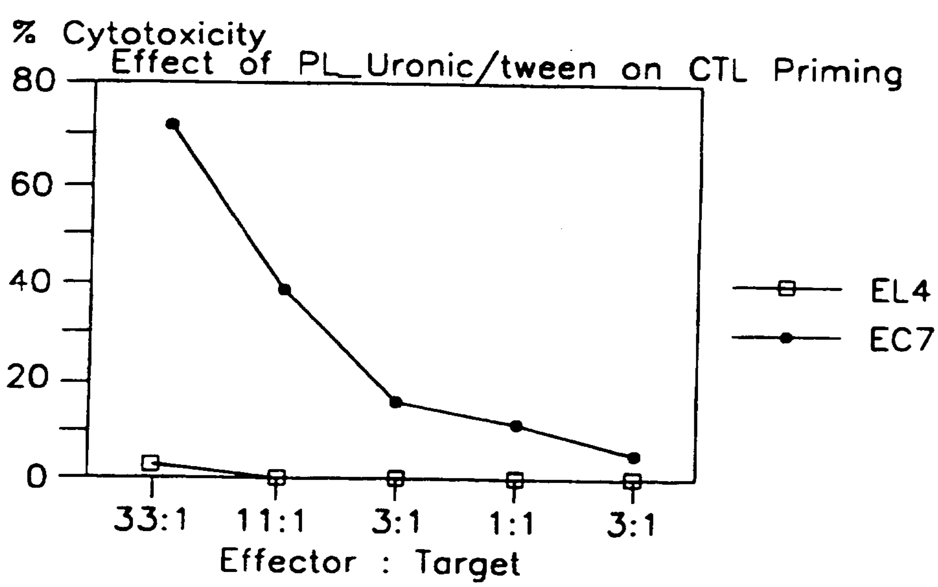

Idox potentiates gp120 induced CTL induction
% Cytotoxicity
30
20
10
0
gp120 in Idox
gp120 in HBSS
11:1
3:1
1:1
3:1
Effector to Target ratio

FIG. 7

FIG. 8


% Cytotoxicity
Effect of PL_Uronic/tween on CTL Priming
80
60
40
20
0
EL4
EC7
33:1
11:1
3:1
1:1
3:1
Effector : Target

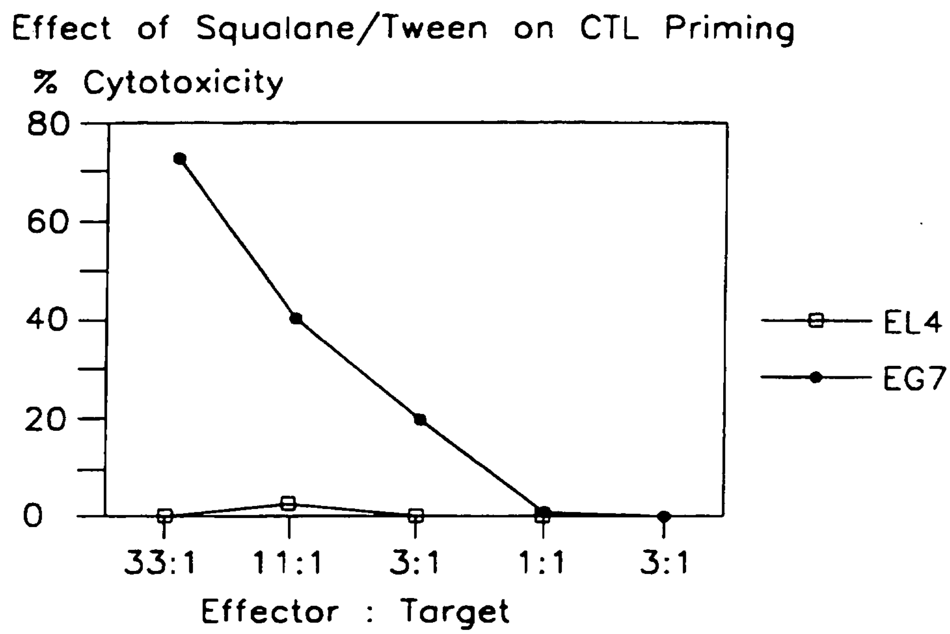

Effect of Squalane/Tween on CTL Priming
% Cytotoxicity
80
60
40
20
0
33:1
11:1
3:1
1:1
3:1
Effector : Target
EL4
EG7

FIG. 9

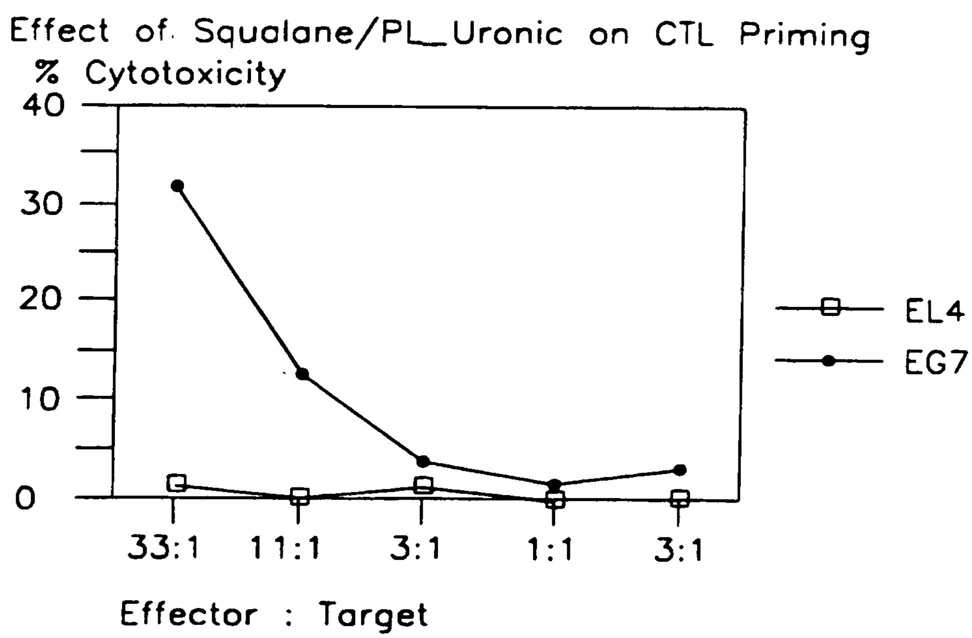

Effect of Squalane/PL_Uronic on CTL Priming
% Cytotoxicity
40
30
20
10
0
33:1
11:1
3:1
1:1
3:1
Effector : Target
EL4
EG7

FIG. 10

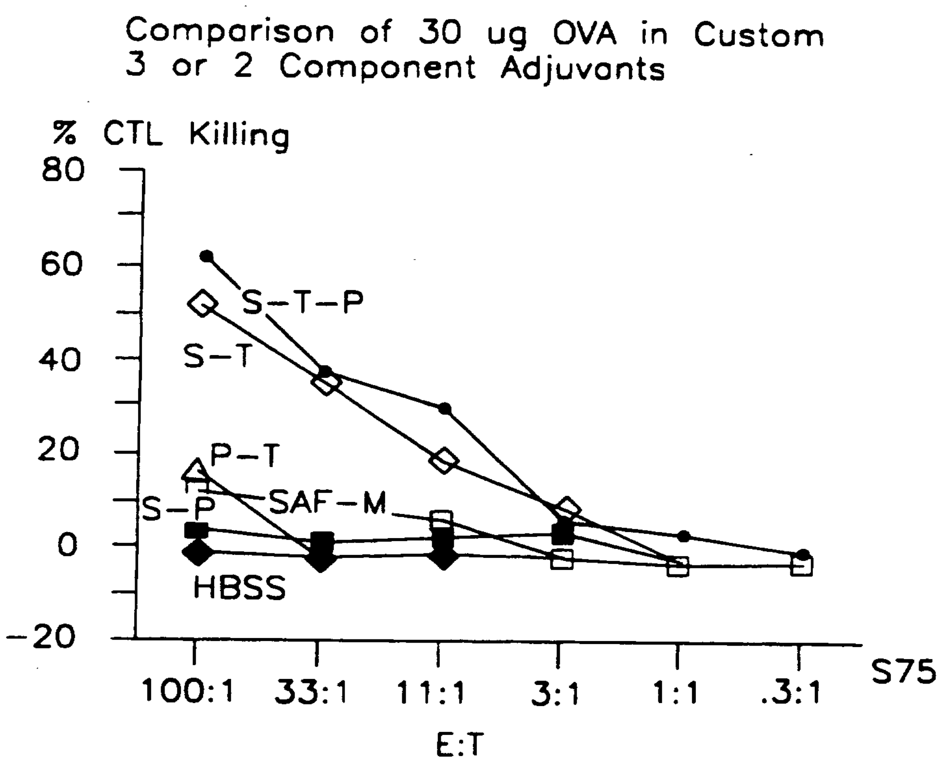

Comparison of 30 ug OVA in Custom
3 or 2 Component Adjuvants
% CTL Killing
80
60
40
20
0
−20
S−T−P
S−T
P−T
S−P
SAF−M
HBSS
100:1
33:1
11:1
3:1
1:1
.3:1
E:T
S75

FIG. 11

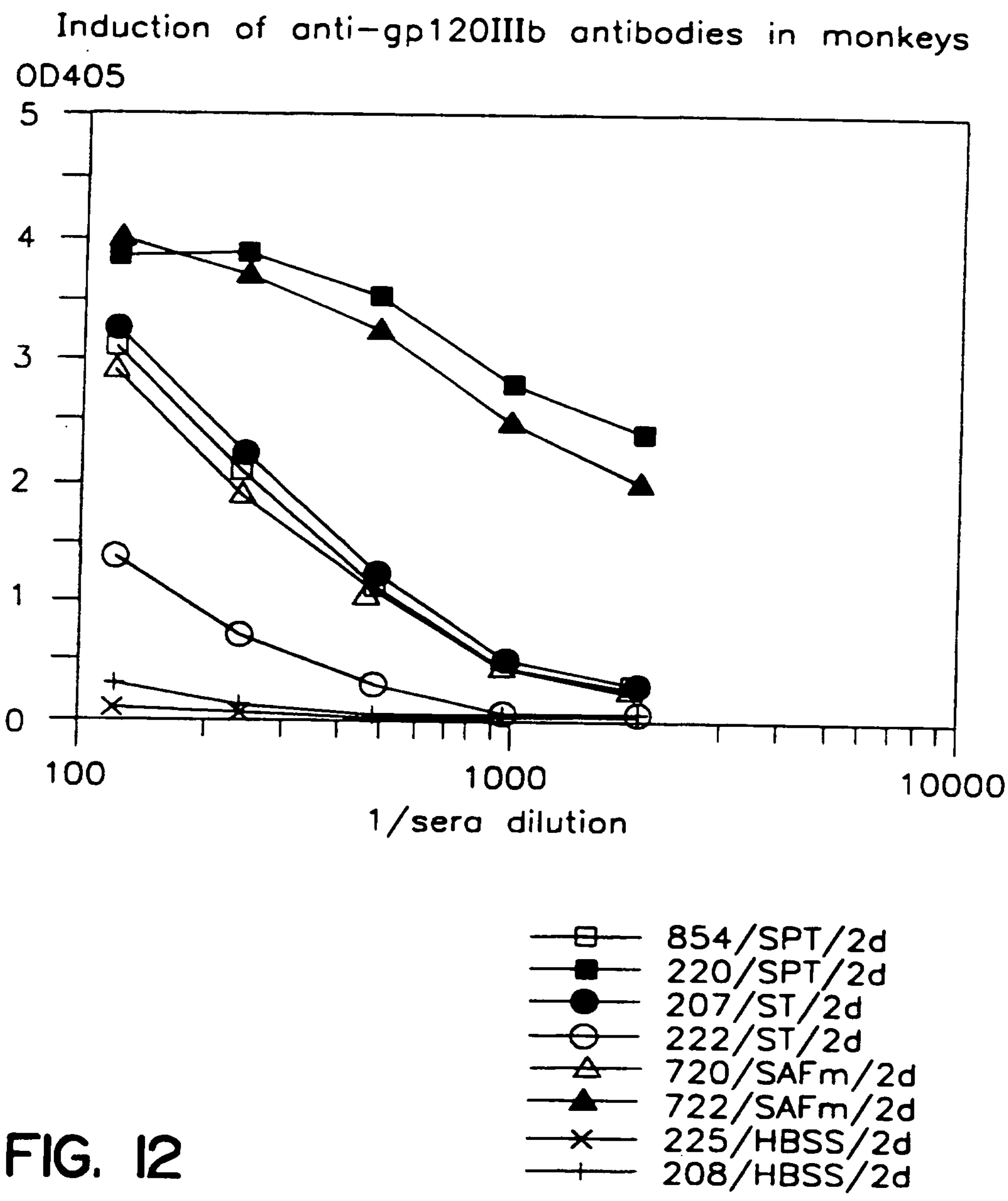

Induction of anti-gp120IIIb antibodies in monkeys
OD405
5
4
3
2
1
0
100
1000
10000
1/sera dilution
854/SPT/2d
220/SPT/2d
207/ST/2d
222/ST/2d
720/SAFm/2d
722/SAFm/2d
225/HBSS/2d
208/HBSS/2d

FIG. 12

Antitumor activity of HOPE2 Cells after a Single Immunization (Day 10) of Soluble E7 Protein in Adjuvant

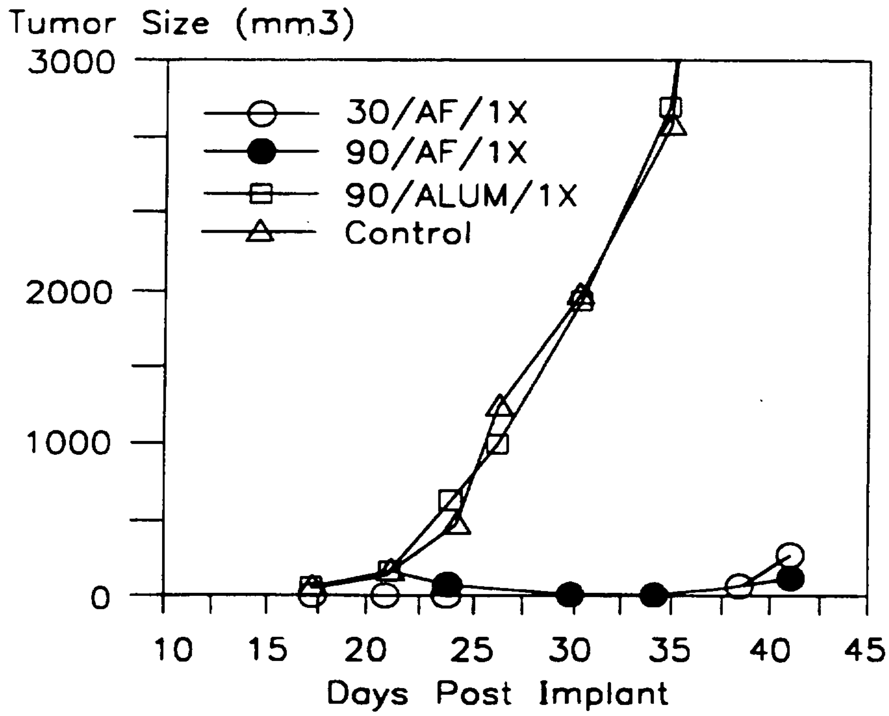


FIG. 13

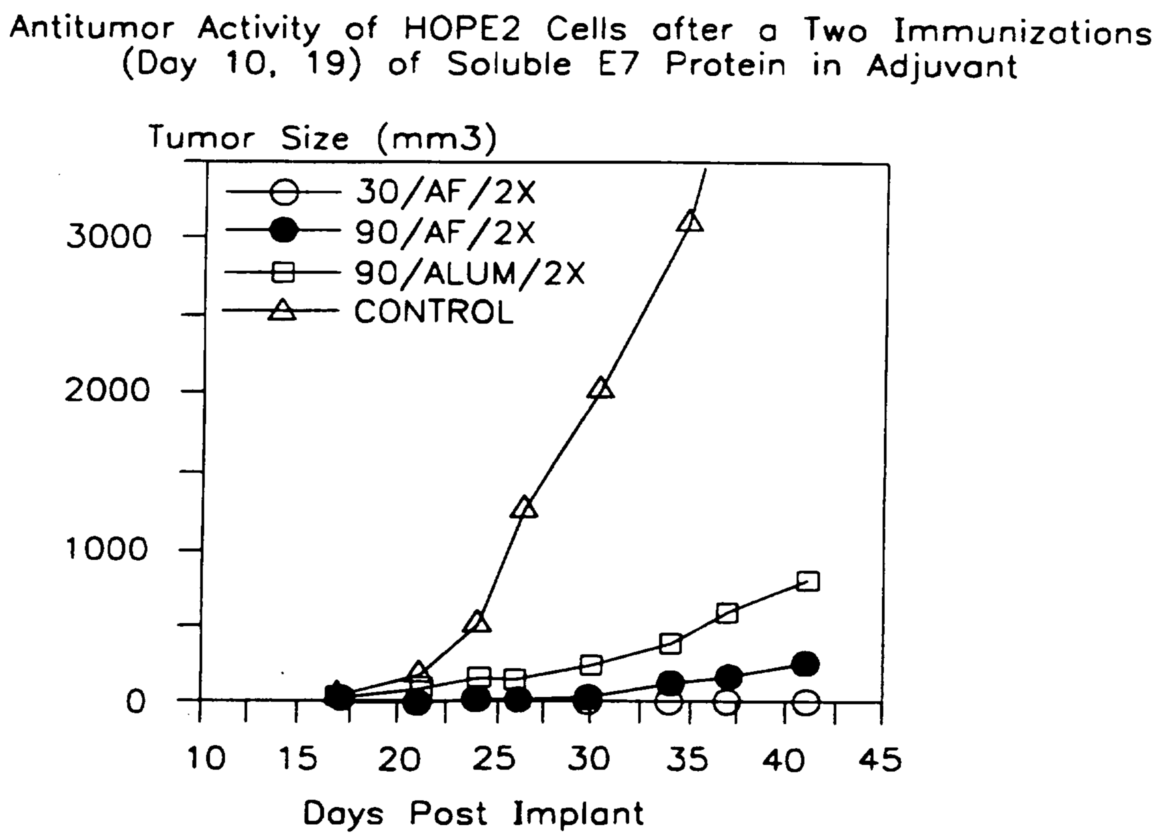

Antitumor Activity of HOPE2 Cells after a Two Immunizations
(Day 10, 19) of Soluble E7 Protein in Adjuvant
Tumor Size (mm3)
30/AF/2X
90/AF/2X
90/ALUM/2X
CONTROL
3000
2000
1000
0
10
15
20
25
30
35
40
45
Days Post Implant

FIG. 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/15433

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/00
US CL :424/450; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 204.1, 207.1, 208.1, 209.1, 210.1, 211.1, 212.1, 278.1, 281.1, 283.1, 450; 530/350, 826

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: BIOSCIENCE, CAPLUS, EMBASE, BIOSIS, TOXLIT, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,234,683 (HUNTER ET AL) 10 August 1993, see entire document.	1-12 and 48-85
X	US, A, 5,114,708 (HUNTER ET AL) 19 May 1992, see entire document.	1-12 and 48-85
Y	US, A, 4,877,611 (CANTRELL) 31 October 1989, see entire document.	1-12 and 48-85
Y	VACCINE, Volume 5, issued September 1987, Byars et al, "Adjuvant Formulation for use in Vaccines to Elicit Both Cell-Mediated and Humoral Immunity", pages 223-228, see entire document.	1-12 and 48-85

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 MARCH 1996	08 MAY 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Deborah Freese /JS JEFFREY STUCKER
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AIDS RESEARCH AND HUMAN RETROVIRUSES, Volume 10, Supplement 2, issued 1994, Hunter et al, "Mechanisms of Action of Nonionic Block Copolymer Adjuvants", pages 595-598, see entire document.	1-12 and 48-85

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/15433

Box I Observations where certain claims wer f und unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-12 and 48-85

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees m t be paid.

Group I, claim(s) 1, 2, 7-12, and 52-85, drawn to a first composition and a first method of using said first composition.
Group II, claim(s) 3-6, 48, and 49, drawn to a second composition.
Group III, claim(s) 13-14, drawn to a method of treating HIV.
Group IV, claim(s) 15-16, drawn to a method of treating malaria.
Group V, claim(s) 17-18, drawn to a method of treating influenza.
Group VI, claim(s) 19-20, drawn to a method of treating hepatitis.
Group VII, claim(s) 21-22, drawn to a method of treating cancer.
Group VIII, claim(s) 23-24, drawn to a method of treating herpes.
Group IX, claim(s) 25-26, drawn to a method of treating RSV.
Group X, claim(s) 27-30, drawn to a method of inducing T-lymphocyte response.
Group XI, claim(s) 31-32, drawn to a second method of treating HIV.
Group XII, claim(s) 33-34, drawn to a second method of treating malaria.
Group XIII, claim(s) 35-36, drawn to a second method of treating influenza.
Group XIV, claim(s) 37-38, drawn to a second method of treating hepatitis.
Group XV, claim(s) 39-40, drawn to a second method of treating cancer.
Group XVI, claim(s) 41-42, drawn to a second method of treating herpes.
Group XVII, claim(s) 43-44, drawn to a second method of treating RSV.
Group XVIII, claim(s) 48-50, drawn to a composition and method of treating cervical cancer.
Group XIX, claim(s) 51 drawn to a method of treating prostate cancer.

Claims 45-47 are generic to claims 25-44.

The inventions listed as Groups I-XIX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The inventions of each of the groups are directed to multiple products and methods of use of the products which are not so linked by a special technical feature within the meaning of PCT Rule 13.2, so as to form a single general inventive concept. The invention of Group I is considered as the main invention in the claims. See PCT Article 17(3)(a) and §1.276(c).